In-house Genotypic Antiretroviral Resistance Test: Optimisation and Validation for Use in Research and Diagnostics.

by

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Thesis presented in fulfilment of the requirements for the degree Master of Medical Sciences (Medical Virology) at the University of Stellenbosch

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part, submitted it at any university for a degree.

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Signature

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Name in full

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Date

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Abstract

It is estimated that 32.8 million people are living with Human Immunodeficiency Virus (HIV) globally with the number of people receiving antiretroviral therapy in low- and middle- income counties increasing to more than 5 million people in 2009. These successes are threatened by treatment failure and the development of resistance to treatment. With an estimated 3.7% patients failing first line treatment after 2 years and 17.9% after 4 years on treatment there is a need for a practical and cheap in-house drug resistance assay that can be used to provide drug resistance data to clinicians and to use as a research tool to investigate drug resistance. In this study we attempted to optimize and validate an in-house drug resistance assay, adapted from Jacobs et al, 2008, to be used as a diagnostic tool and to study the presence of antiretroviral resistance in patients on the Western Cape Mother-To-Child-Transmission (MTCT) regimen.

Quality control samples were received from The National Institute of Communicable Diseases AIDS Virus Research Unit, The Round Robin HIV-1 genotyping assessment system from the University of Würzburg and the QCMD assessment system were used for the optimization and validation of an in-house drug resistance assay. The ViroSeq™ HIV-1 Genotyping System was used for comparison of sample and mutation detection.

It was possible to optimise and validate a genotyping assay for diagnostic testing and research use by comparison with the ViroSeq™ HIV-1 Genotyping System and evaluation with external quality assessment systems. This assay could subsequently be used to determine the development of genotypic-antiretroviral resistance in patients treated according to the provincial prevention of mother-to-child-transmission (PMTCT) protocol in the Western Cape (single dose nevirapine (sd-NVP), combined with a short course Zidovudine (AZT)). Patient samples were collected from pregnant women who took part in the Western Cape PMTCT program and visited the Tygerberg Obstetrics Clinic and Delft Community Hospital. EDTA blood was obtained to measure CD4-cell count, viral load, and to do genotyping for viral subtype and the presence of resistance mutations. Information on prior exposure to antiretroviral therapy was also collected. A detected resistance rate of 17.1% in this

predominantly HIV-1 subtype C population is lower than previously recorded when sd-NVP was administered to HIV-1 subtype C positive patients in PMTCT programs. This could indicate that a dual PMTCT regimen including AZT and NVP reduces the risk of resistance to NVP relative to a regimen that uses sd-NVP.

The genotyping assay uses four primers to amplify the PR and the RT gene separately to obtain PCR products, of 487 and 804 base pairs respectively for sequencing. The two PCR products were sequenced with three and five primers respectively to sequence the complete PR and approximately 250 amino acids of the RT gene. The sequences generated, thus, are analysed and aligned with the Sequencer V4.7 software to obtain a consensus sequence of approximately 1200 base pairs for analysis of resistance mutations in the *protease* and *reverse transcriptase* genes.

The developed assay was hence further simplified and improved, by combining the PR and RT assay into one, which was optimised and validated for use in the routine diagnostic setting. The final genotyping assay uses 8 primers for sequencing to obtain a 1200 bp sequence for genotyping that contains the protease and the 5' of the reverse transcriptase genes in which antiretroviral resistance associated mutations are found. The assay was accredited by SANAS in 2008.

Abbreviations

3TC	Lamivudine
d4T	Stavudine
ºC	Degree Celsius
µl	Microlitre
µM	Micromolar
AIDS	Acquired Immunodeficiency Syndrome
AMV	Avian myeloblastosis virus
ART	Antiretroviral treatment
AZT	Zidovudine
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
CA	Capsid
cDNA	Complementary DNA
copies/ml	Copies per millilitre
CRF	Circulating recombinant forms
DNA	Deoxyribonucleic acid
DTT	Dithiotheritol
ddi	Didanosine
ddNTP	Dideoxynucleoside triphosphates
dNTPs	Deoxynucleotide triphosphates
dNTPs A,G,C,T	Adenine, Guanine, Cytosine, Thymidine
ds-DNA	Double stranded-DNA
EDTA	Ethylene diamine tetra-acetic acid
EFV	Efavirenz
env	Envelope gene

ENV	Envelope protein
EQA	External Quality Assessment
FDA	Federal and Drug Administration
FTC	Emtricitabine
gag	Group antigen gene
Gag	Group antigen protein
GART	Genotypic antiretroviral resistance testing
GDR	Genotypic drug resistance assays
Gp	glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HIVNET	Human immunodeficiency virus Network for Prevention Trials
IIH	Inproved In-house assay
jpHMM	Jumping profile Hidden Markov Model
kg	Kilogram
LAV	Lymphadenopathy-associated virus
LDL	Lower than the detection limit
LPV/r	Lopinavir/ritonavir
M	Molar
MA	Matrix
Mg+	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_2$	Magnesium sulphide

MHC	Major hitocompatibility
mM	MilliMolar
ml	Millilitre
MTCT	Mother-To-Child-Transmission
n	Number
NAMs	nucleoside analog-resistance mutations
NC	Nucleocapsid
nef	Negative factor gene
Neg/N	Negative control
Nef	Negative factor protein
NHLS	National Health Laboratory Service
ng	Nanogram
NICD	National Institute Communicable Diseases
nm	Nanometre
NNRTI	Non-nucleoside analogue Reverse Transciptase Inhibitor
NRTI	Nucleoside analogue Reverse Transciptase Inhibitor
NTC	Non-Template Control
NVP	Nevirapine
PACTG	Paediatric AIDS Clinical trial group
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PI	Protease inhibitor
PIC	Pre-integration complexes
pmol	Picomoles
PMTCT	Prevention of Mother To Child Transmission
pol	Polymerase enzyme

Pol	Polymerase protein
PR	Protease enzyme
PT	Proficiency Testing
QCMD	Quality Control for Molecular Diagnostics
rev	Regulatory of viral expression gene
Rev	Regulatory of viral expression protein
RNA.	Ribonucleic acid
RT	Reverse Transcriptase enzyme
RT-PCR	Reverse transcriptase Polymerase Chain Reaction
RTC	Reverse transcriptase complexes
SANAS	South African National Accreditation System
sd-NVP	Single dose Nevirapine
SIV	Simian immunodeficiency virus
TAC	Treatment Action Campaign
TAMs	Thymidine analog mutations
Taq	*Thermus aquaticus*
tat	Transcriptional transactivator gene
Tat	Transcriptional transactivator protein
TDF	Tenofovir
T*fl*	*Thermus flavus*
TFIH	Two fragment in-house assay
™	Trade Mark
vif	Virion infectivity factor gene
Vif	Virion infectivity factor protein
VL	Viral load
vpr	Viral protein R gene

Vpr	Viral protein R protein
vpu	Viral protein U gene
Vpu	Viral protein U protein
U	Units
UK	United Kingdom
URF	Unique recombinant forms
USA	United State of America
QC	Quality control
QCMD	Quality Control of Molecular Diagnostic system

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References

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Van Zyl GU, Claassen M, Engelbrecht S, Laten JD, Cotton MF, Theron GB, Preiser W. Zidovudine With Nevirapine for the Prevention of HIV Mother-to-Child Transmission Reduces Nevirapine Resistance in Mothers From the Western Cape, South Africa. J Med Virol. 2008: 80(6):942-6.

Chapter 1

Chapter 1

1. Introduction and literature review

1.1 Introduction

In 2008, an estimated 32.8 million people were living with human immunodeficiency virus (HIV) globally with 2.7 million new infections. In South Africa 5.7 million people are living with HIV, of which almost one in three are women attending public antenatal clinics (UNAIDS, 2010). The number of people receiving antiretroviral therapy in developing countries have increased to 5.2 million in 2009 and it is expected that treatment access have averted 14.4 million acquired immunodeficiency syndrome (AIDS) related deaths since 1996 (UNAIDS 2010). Unfortunately some patients have treatment failure and resistance to the initial treatment regimen. In a study done by Boulle *et al*, 2008 it was estimated that 3.7% fail first line treatment after 2 years and 17.9% after 4 years on treatment (Boulle *et al*, 2008). The cost of second line antiretroviral drugs still remains too high for use in many developing countries with an increasing number of patients that requires it (UNAIDS 2010).

Since most of HIV infected people are living in low-income countries the affordability of anti-retroviral drugs and monitoring of patients receiving treatment, is an important factor. With the efforts of treatment activists the prices of anti-retroviral drugs have decreased but the cost of the new most tolerable combination therapy regimens is still very high. In resource limited settings the most affordable treatment regimens are often preferred. Treatment failure increases costs as more expensive second line regimens are then needed to suppress viral replication and because failing patients have an increased risk of suffering from HIV related conditions. Drug resistance testing can help to lower cost by enabling clinicians to choose the most effective treatment regimen and could avoid unnecessary switches to second-line therapy in patients without resistance, but the cost of drug resistance testing, with commercial assays, is extremely high. Licensed commercial assays that are available are the ViroSeq™ HIV-1 Genotyping System (Celera Diagnostics, Alameda, California, USA) and the TRUGENE™ HIV-1 Genotyping Kit assay (Visible Genetics, Toronto, Canada). These are routinely used for diagnostic HIV drug resistance testing in industrialised countries but the cost is prohibitive for resource-

limited settings. There is therefore a need for a practical and affordable in-house antiretroviral drug resistance assay for clinical and research use.

In this study we will attempt to optimize and validate an in-house drug resistance assay, adapted from Plantier *et al* (2005) and published in Jacobs *et al* (2008), for clinical diagnostic use and for the study of antiretroviral resistance in patients on the Western Cape Mother-To-Child-Transmission (MTCT) regimen.

This chapter will give a short overview of the history of AIDS and HIV, the epidemiology of the virus, the genomic structure, proteins, and the replication of the virus, viral diversity, transmission of the virus, treatment of the infection, drug resistant development and mutations, assays available for drug resistance testing and the aim of this study.

1.2 Prevalence of HIV and availability of treatment

Of the estimated 32.8 million people that are living with HIV, the largest HIV positive population, 22.5 million are living in Sub-Saharan Africa. The number of new infections in 2009 was estimated to be 2.6 million and AIDS related deaths were 1.8 million of which 31% of new infections and 34% of deaths due to AIDS related illness occurred in Sub-Saharan Africa. It has been estimated that the global HIV prevalence have been level since 2001 and that most of Sub-Saharan Africa's HIV prevalence have stabilised or are showing a decline. These declines could be due to the natural history of the pandemic or a drop in transmission due to interventions such as increased access to antiretroviral therapy in the region.

The availability of anti-retroviral therapy to those who requiring treatment have increased 10 fold from 2002 and the number of people receiving antiretroviral therapy in developing countries increased to more than 5 million people in 2009. It is estimated that in developing countries, only 36% of those who need treatment are receiving it. In South Africa approximately 5.6 million people are living with HIV, of which 991 556 are receiving antiretroviral therapy in 2009. Almost one in three people in South Africa infected are women attending public antenatal clinics. South Africa is one of the countries that showed a marked increase in the availability of treatment for HIV positive pregnant women. The coverage of prevention of mother-to-child-transmission increased from 15% to more than 80% from 2004 to 2009 (UNAIDS 2010, Republic of South Africa, 2008).

1.3 A short overview of the history of HIV and AIDS

AIDS was first recognized as a new disease in 1981, with the report of 4 cases of *Pneumocystis carinii* pneumonia in previously healthy homosexual men with a distinct and unusual clinical syndrome, which was linked to a severe acquired T-cell defect (Gottlieb *et al*, 1981). Other cases of opportunistic infections or tumours such as kaposi sarcoma, in homosexual men, provided additional evidence of acquired immunodeficiency, which led investigators to suspect a new pathogen that could be sexually transmitted (Centre for Disease Control, 1982, Masur *et al*, 1982). In 1983 HIV-1, called lymphadenopathy-associated virus (LAV), was isolated from lymph node cells of a patient with lymphadenopathy (Barré-Sinoussi *et al*, 1983) and the pathogen was identified for the first time. It was found to be transmitted through sexual intercourse, via blood and blood products and from mothers to infants, either before, during and after birth or through breast feeding (Cohen *et al*, 2008). In 1985 a second virus that causes AIDS, HIV-2, was identified in West African patients (Barin *et al*, 1985).

1.4 The Origin of HIV

It is believed that HIV originated in Africa. Phylogenetic evidence was presented that showed that HIV-1 crossed over from the chimpanzee *Pan troglodytes troglodytes* (Gao *et al*, 1999) and HIV-2 from the sooty mangabey *Cercocebus atys* (Chen *et al*, 1996; Chen *et al*, 1997). The first known human HIV-1 infection, based on the detection of antibodies from a frozen serum sample, occurred in Central Africa in 1959 (Nahmias *et al*, 1986) and phylogenetic analytical methods have indicated that the virus originated before 1930 (Korber *et al,* 2000, Salemi *et al*, 2000, Worobey *et al*, 2008). The epidemic started in Africa and spread to the homosexual population in America and Europe with newer information indicating that the epidemic spread from Central Africa via Haiti to America and Europe (Gilbert *et al*, 2007).

It was believed to be a problem of men who have sex with men and intravenous drug users. It soon became clear that the disease was spreading to the heterosexual populations as well as infants, haemophiliacs, and blood recipients (Centre for Disease Control, 1986 and Scott *et al*, 1984). Today the virus has spread worldwide and affects every country to different degrees, reflecting biological and social factors (Cohen *et al*, 2008).

1.5 HIV genome, proteins, morphology, and replication

The HIV genome, proteins, morphology and replication are reviewed by the following publications: Briggs *et al*, 2003, Frankel and Young, 1998. Freed, 2001, Nisole and Saib, 2004, Sierra *et al*, 2005, Turner and Summers, 1999, Wang *et al*, 2000.

1.5.1 HIV-1 genome and proteins

The HIV-1 genome contains two copies genomic ribonucleic acid (RNA). The RNA genome is approximately 9.2-kilobases and it contains 9 open reading frames that encode for different viral proteins. The genome is presented schematically in Figure 1.1

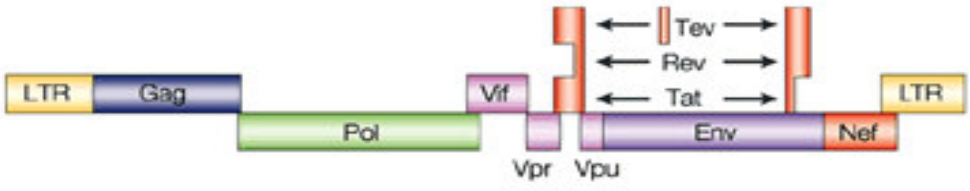

Figure 1.1: Schematic diagram of the HIV-1 genome (Wikipedia. HIV genome.png http://en.wikipedia.org/wiki/Image:HIV_genome.png 24 November 2010).

The structural genes are the *gag*, *env*, and *pol* genes that are present in all retroviruses. The *gag* gene codes for the virus core proteins, the capsid that packages the viral RNA, the matrix that contains nuclear import signals and the nucleocapsid that binds RNA. The *pol* gene codes for the enzymes, protease (PR), reverse transcriptase (RT), RNase H, and integrase. The *env* gene codes for virus envelope glycoproteins gp120 on the surface and transmembrane glycoproteins gp41. The *tat* and *rev* genes are essential for replication *in vitro*. *Tat* is responsible for the regulation of viral transcription and *rev* regulates viral RNA transport and splicing. The *nef, vpu, vif, vpr* gene are not essential for replication in vitro and are called the accessory genes. The *nef* gene down regulates the CD4 and Major histocompatibility-I (MHC-I) and binds cellular kinases. It plays an essential role in the induction of disease. The *vpu* gene down regulates the CD4 and MHC-I and promotes virus release. The *vif* gene facilitates the viral replication and virion maturation. The *vpr* gene plays a role in the regulation of viral and cell gene expression and blocks cell proliferation. A summary of the HIV-1 viral proteins and their functions are set out in Table 1.1 and ribbon representations of the structurally characterized viral proteins and protein fragments are set out in Figure 1.2.

Table 1.1: The HIV-1 viral proteins and their functions (Frankel and Young, 1998)

Protein	Function
Capsid (CA)	Package the viral RNA
Matrix (MA)	Contains nuclear import signals
Nucleocapsid (NC)	Helps with incorporation of Vpr during viral assembly
gp120 surface envelope glygoprotein	Binding of the viral particle to the host cell receptors
gp41 transmembrane glycoprotein	Fusion with the host cell and viral entry
Tat	The regulation of viral transcription
Rev	Regulates viral RNA transport and splicing
Nef	Down regulates the CD4 and MHC-I and binds cellular kinases, enhances viral particle infectivity, effects on cellular signal transduction and activation
Vif	Facilitates the viral replication and virion maturation
Vpr	Plays a roll in the regulation of viral and cell gene expression and blocks cell proliferation
Vpu	Down regulates the CD4 and MHC-I and promotes virus release

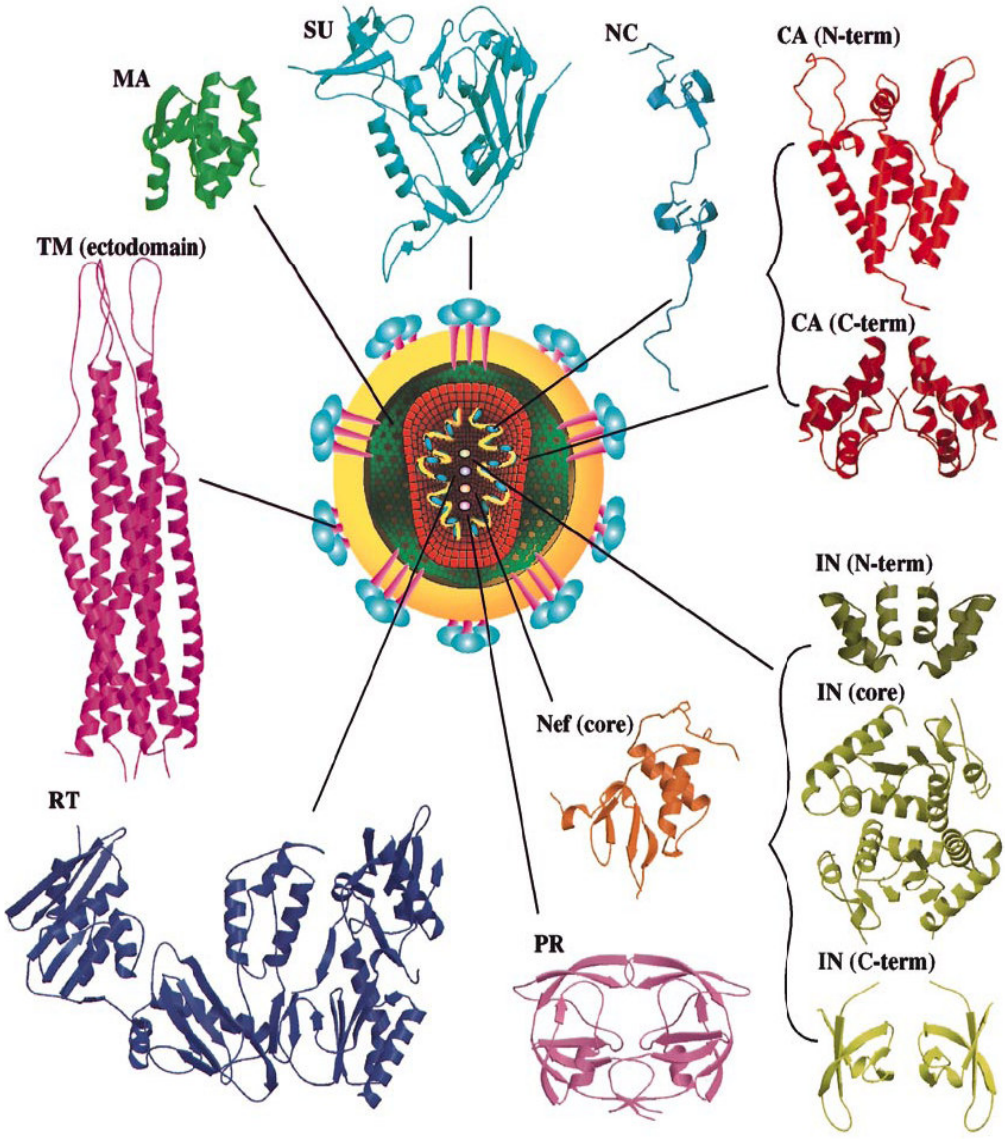

Figure 1.2: Drawing of the mature HIV virion surrounded by ribbon representations of the structurally characterized viral proteins and protein fragments. The protein structures have been drawn to the same scale (Turner and Summers, 1999).

1.5.2 HIV-1 morphology

The HIV-1 virus is a *Lentivirus* from the subfamily of *Orthoretrovirinae, Family Retroviridae.* A diagram of the HIV-1 particle is presented in Figure 1.3.

The HIV-1 virus contains a lipid bilayer envelope that is derived from the membrane of the host cell. The lipid membrane contains approximately 72 spikes of viral Envelope protein (Env) glycoproteins and cellular proteins. The surface glycoprotein, gp120 is anchored to the virus particle by interaction with the transmembrane glycoprotein, gp41. Other cellular membrane proteins are also present, including actin

and ubiquitin. The core is formed by approximately 2000 copies of the matrix protein directly to the inside of viral envelope. In the centre of the viral particle is a conical shaped capsid, capsomeres are assembled in hexametric rings that contain approximately 2000 copies of capsid protein. The capsid contains two copies of the viral RNA. The genome is stabilized by forming a complex with nucleocapsid protein. The capsid also contains the viral enzymes, protease, reverse transcriptase, and integrase. The accessory proteins Negative factor protein (Nef), viron infectivity factor protein (Vif) and Viral protein (Vpr) are also found enclosed in the capsid.

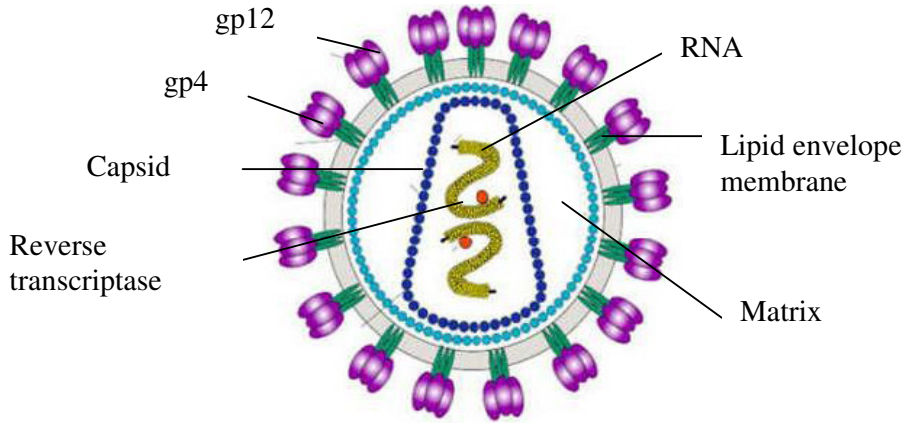

Figure 1.3: Schematic diagram of the HIV-1 particle modified from Wikipedia. http://commons.wikimedia.org/wiki/Image:Niaid-hiv-virion-mod.jpg 24 November 2010.

1.5.3 HIV-1 replication

The general HIV-1 replication features are set out in Figure 1.4. HIV infects a large amount of cells in the immune system. CD4+ T-cells, Cytotoxic T-lymphocytes, CD4+ monocytes, macrophages, CD4+ dendritic cells and B cells can all be infected by HIV and resting CD4+ T cells are the major reservoirs for latent virus (Blankson *et al*, 2002). The virus have been found in the blood plasma, peripheral blood mononuclear cells (PBMC's), lymph nodes, breast milk, the central nervous system, the genitourinary tract and other body fluids and cells after infection (Blankson *et al*, 2002, Coovadia, 2004, Pierson *et al*, 2000, and Stebbing *et al*, 2004).

The life cycle can be divided into 2 phases. The early phase begins with the recognition of the host cell by the virus particle and includes all processes up to the point and including the integration of the viral genomic DNA into the host

chromosome. The late phase begins with the synthesis of RNA and includes all processes to the stage where the mature virus leaves the cell.

During the early phase the virus attach to the surface of the host cell by the interaction of the gp120 protein with the CD4 receptor. This interaction triggers a change in the gp120 that opens a previously hidden region that acts as a co-receptor binding site. CXCR4 and CCR5 have been identified as the most important co-receptors necessary for viral entry and replication into the host cell and will bind to the opened site by electrostatic interactions. Other co-receptors that can be used by HIV-1 include CCR3, CCR2b, CCR8, CCR9, Bonzo/STRL33, BOB/GPR15 (Berger *et al*, 1999). The binding to a cellular co-receptor triggers another change of the Env protein and the HR1 and HR2 domains of the gp41 will interact with one another and form a six-helix bundle structure that contains the "fusion peptide", a highly hydrophobic N terminus, that will initiate the membrane fusion. The viral capsid will enter the host cytoplasm after fusion between the viral and the host membrane.

The uncoating of the core is not well understood. It has been found that cellular proteins and capsid phosphorylation play a role in uncoating (Wacharaporini *et al*, 2007 and Auewarakul *et al*, 2005). After the uncoating of the core, the reverse transcriptase complexes (RTC) are formed, followed by the pre-integration complexes (PIC). The uncoating of the core initiates the reverse transcription process by which RNA is transcribed into complementary deoxyribonucleic acid (DNA) by the enzyme Reverse Transcriptase (RT). The viral DNA is transported to the nucleus as part of the PIC that includes the integrase, matrix, RT and Vpr proteins. The PIC enters the nucleus via the nuclear pore complexes, large supramolecular protein structures, with the help of the Vpr protein.

Integration of linear double stranded DNA into the host DNA is mediated by the integrase enzyme. The sites for integration in host DNA are random and integrase creates staggered ends in the linear double stranded DNA by removing several nucleotides from the 3' termini of both strands of DNA. This molecule is inserted into the host DNA that has been cleaved asymmetrically.

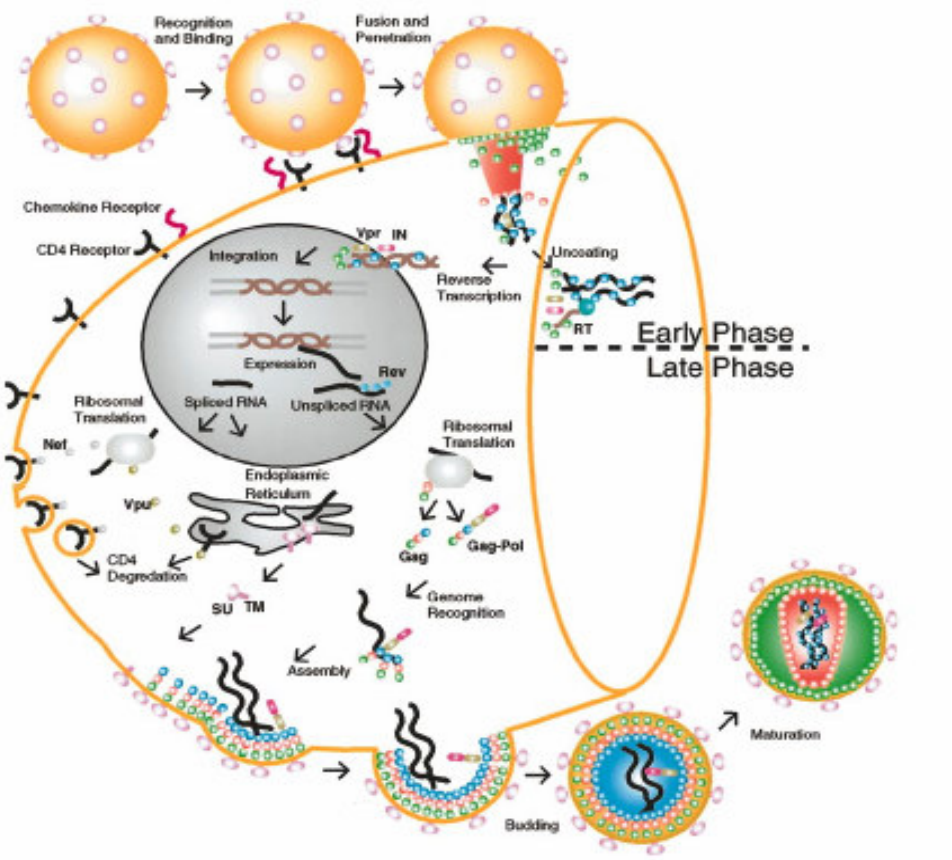

Figure 1.4: General features of the HIV-1 replication cycle (Turner and Summers, 1999).

The late phase of the virus replication begins with the transcription of the viral RNAs, which will encode the structural, regulatory, and accessory proteins for viral replication from the integrated proviral DNA by cellular RNA polymerase II. The first RNAs synthesised encode for the regulatory proteins Tat, Rev and Nef. The Tat protein acts as a transcriptional activator. Unspliced mRNAs are present in the nucleus where they are processed. The Rev protein mediates the export of the full length unspliced HIV mRNAs, encoding for the Gag and Gag-Pol proteins, and single spliced HIV mRNAs, encoding for the Env, Vpu, Vif, Vpr proteins, to the cytoplasm for synthesis and packaging.

In the cytoplasm the spliced *env* mRNA gene is translated to the Env precursor protein, gp160, which is glycosylated in the endoplasmic reticulum. The *gag-pol* gene is translated to the Gag and the Gag-Pol precursor proteins. The Gag-Pol precursor polyprotein is produced with the occasional ribosomal 1 frame shift during translation. The Gag-precursor polyprotein is cleaved by the protease enzyme to

produce 6 structural proteins which rearrange and produce the mature viral particle. After translation the Env proteins migrate and insert in the plasma membrane. The Gag and Gag-Pol polyproteins move to the cellular membrane and start to assemble directed by the Gag polyprotein. Viral enzymes, full size genomic RNA, cellular tRNAlys3 primer and cellular compounds are assembled within the immature viral particle. This viral particle buds through the plasma membrane producing immature viral particles. During the budding process the protease enzyme is activated and cleavage of the Gag and Gag-Pol polyproteins are activated that release the structural proteins and enzymes and the mature viral particle is formed. The infection of resting memory or naïve $CD4^+$ T cells, macrophages and mononuclear cells leads to latent infection.

1.6 The HIV-1 enzymes that is essential to this project

The *pol* gene of the HIV-1 genome encodes for the viral enzymes, protease (PR), reverse transcriptase (RT), RNase H, and integrase. These enzymes are produced by the cleavage of the Gag-Pol polyprotein. These enzymes have been reviewed by the following publications: Fields *et al*, 1996, Frankel and Young, 1998, Meek and Dreyer, 1990, Prasad and Goff, 1990, Shafer, 2002 and 2004, Turner and Summers, 1999

1.6.1 The Protease enzyme

The PR is a dimer that consists of two structurally identical monomers which each are 99 amino acids long. A structural model of HIV-1 PR homodimer is presented in Figure 1.5. The enzyme targets the cleavage sites on the Gag and Gag-Pol polyproteins to produce the structural proteins and enzymes of the virus. The active site in each monomer is positioned in a loop at positions 25-27, and forms part of the catalytic site. Above the active site is a binding cleft for the substrate. There is enough room in the cleft to accommodate a substrate of seven amino acids in length. The entrance to the binding site is covered by to flaps in the dimer and these flaps move to permit substrate to enter. The enzyme has specificity for more than one cleavage site. There are four cleavage sites in the Gag and seven in the Gag-Pol polyprotein. Cleavage specificity is determined by four amino acids upstream and three amino acids downs stream in the target substrate. The amino acids upstream of the cleavage site are always hydrophobic and unbranched at the β-carbon. The enzyme cleave 9

different peptide sequences to produce the matrix, capsid, nucleocapsid, and p6 protein from the Gag polyprotein and protease, reverse transcriptase, RNase H and integrase proteins from the Gag-Pol polyprotein.

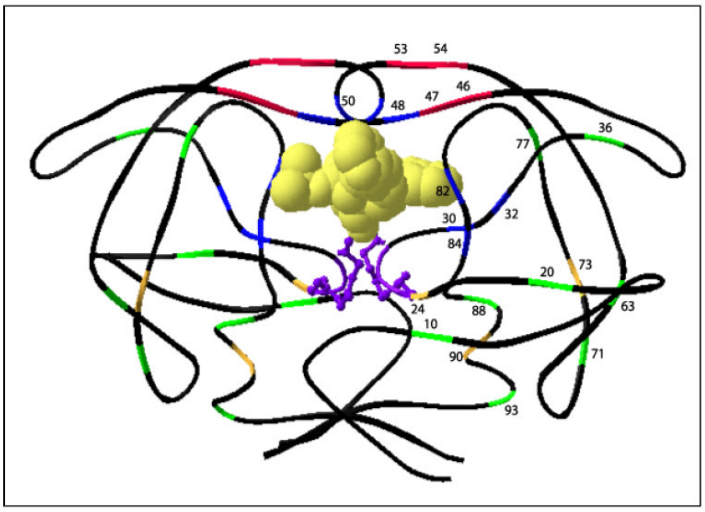

Figure 1.5: Structural Model of HIV-1 Protease Homodimer The polypeptide backbone of both protease subunits is shown. The active site, made up of positions 25-27 from both subunits, is displayed in ball and stick mode. The protease inhibitor resistance mutations are shown for the subunit on the left but not for the mirror-image subunit on the right. The protease was co-crystallized with indinavir, which is displayed in space-fill mode. This drawing is based on a structure published by Chen *et al*, 1994.

1.6.2 The Reverse Transcriptase (RT)

A structural model of HIV-1 RT enzyme is presented in Figure 1.6. The RT is a RNA-dependant DNA polymerase and can synthesize DNA from both RNA and DNA templates. The PR enzyme cleaves the RT enzyme from the Gag-Pol polyprotein during viral assembly. This cleavage happens in two steps. First the p66 is cleaved from the polyprotein and forms a homodimer. The p66 subunit is cleaved near the C-terminus to yield a heterodimer that contains both the p51 and the p66. The p51 subunit contains 440 amino acids of the RT gene and has no enzymatic activity. The p66 subunit contains the complete 560 amino acids of the RT gene. The N termini of both subunits are identical. The p51 and p66 interact in a head-to-tail configuration to produce the heterodimer. The structure of the RT domain has 5 subdomains that resemble a hand with fingers, palm and thumb. The catalytic site of the RT lies in the cleft of the palm of the p66 subunit. The thumb of the p66 subunit contains two α-helices that together with the palm act as a clamp to position the template-primer to

the active site of the enzyme. The 3-hydroxyl of the primer terminus is in position for polymerization of the incoming nucleotide triphosphate.

RT is responsible for HIV-1 diversity. It has poor proofreading ability and non-complimentary incorporation of nucleotides occurs that leads to single point mutations. Slippage of the two DNA strands may occur that will lead to deletions or insertions of one and more nucleotides. A frameshift can occur when misincorporation is followed by misalignment. Base substitutions can result when slippage occur with correct insertion and realignment. It can also cross over from one template to another resulting in recombination (McCutchan, 2006). All of these can produce variation over a large portion of the genome and contribute to diversity and mutations that can lead to resistance.

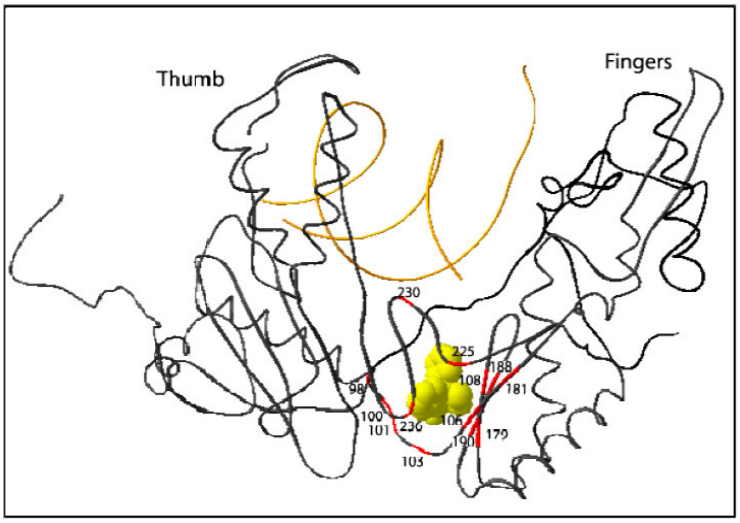

Figure 1.6: Structural model of HIV-1 reverse transcriptase (RT). The polypeptide backbone of the "fingers", "palm", and "thumb" subdomains of the p66 subunit are shown. This drawing shows the RT that is co-crystallized with nevirapine, which is displayed in space-fill mode. The active site is shown in ball and stick mode. The positions associated with Non-nucleoside reverse trancriptase inhibitor (NNRTI) resistance are shown surrounding the hydrophobic pocket to which nevirapine and other NNRTIs bind. The drawing is based on a structure published by Kohlstaedt *et al*, 1992.

1.6.3 RNase H

RNAse H functions in reverse transcription by degrading the RNA of RNA:DNA hybrids and uncovering the template for viral DNA synthesis. RNAse H also produces oligoribonucleotide primers for the reverse transcriptase process. RNAse H is cleaved from the Gag-Pol polyprotein by PR on the p66 subunit of the RT enzyme. The viral

RNAse H is folded into a five-stranded mixed β-sheet flanked by an asymmetric distribution of four α-helices. Four acidic amino acid residues in the RNAse H enzyme of HIV-1 have been identified as necessary for the catalytic activity of the enzyme. The RNase H only becomes catalytically active on addition of the p51 subunit.

1.6.4 Integrase

The integrase enzyme possesses both DNA cleavage and joining activities. A pharmacophore model for the integrase enzyme is presented in Figure 1.7. The enzyme is responsible for the linkage of double-stranded viral DNA into the host cell genome. The C-terminus of the Gag-Pol polyprotein is cleaved by the PR enzyme to produce the integrase enzyme. The core domain consists of a central five-stranded β-sheet and six helices. The enzyme forms a dimer – two monomers of integrase are bound together. Each molecule of integrase contains one catalytic site and has separate DNA binding sites for the ends of linear viral DNA and double-stranded host cell DNA. The substrate of the enzyme is short double-stranded oligonucleotides corresponding to termini, called *att* sites of linear viral DNA. The activities of the integrase gene include the cleavage of deoxythymidylate-deoxythymidylate (TT) dinucleotide from the 3' of double-stranded *att* site substrate, the cleavage of double-stranded target DNA to produce a staggered 5 base overhang and the transfer of activity in which the recessed 3' end in the substrate DNA is joined to the 5' phosphoryl end in the target DNA.

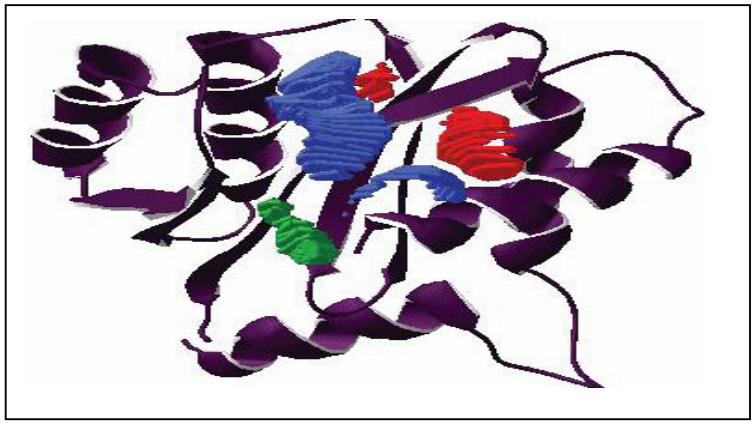

Figure 1.7: The pharmacophore model for the integrase enzyme. Jaganatharaja and Gowthaman, 2006.

1.7 HIV Diversity

There are two genetically distinct HIV types identified, HIV-1 is associated with disease across the globe and HIV-2 is associated with disease mostly in western Africa. The two viruses are similar in the pathogenesis and both lead to AIDS related death, although HIV-2 is associated with a slower disease progression. (Klimas *et al*, 2008). Phylogenetic analyses have shown that there are four distinct groups of HIV-1, HIV-1 group M (major), group O (outlier), group N (non-M/non-O) and group P. (Kober *et al*, 2000, Keele *et al*, 2006, Plantier *et al*, 2009). These groups can be further divided into subtypes on the bases of the phylogenetic relation. Group M is responsible for the majority of HIV-1 infection worldwide. It can be subdivided into subtypes A to K. Two of these subtypes can be divided into sub-subtypes, A1 to A4, F1 and F2. Recombinant forms have also been described (McCutchan, 2006, Ramirez *et al*, 2008, Taylor *et al*, 2008,) and can be divided into circulating recombinant forms (CRF) and unique recombinant forms (URF). There are 48 CRF (Los Alamos Sequence Database, 2010). These have emerged when genomes of different subtypes combined to form a genetically distinct viral lineage. Recombination occurs when a patient is infected with two different HIV-1 subtypes. During the replication of the viruses an RNA stand from the one virus is co-packaged with an RNA stand from the other when a new viral particle is formed. When a new replication cycle starts, the two RNA strands of the virus are transcribed to proviral DNA. The reverse transcriptase enzyme switches between the RNA templates to give rise to recombinant proviral DNA that is a mixture of the two RNA strands containing genes from both the HIV subtypes infecting the patient (Ramirez *et al*, 2008, Taylor *et al*, 2008). The CRF represent recombinant forms that are circulating in the population, so that it could be detected in epidemiologically unlinked cases. URF are recombinants with a unique structure and subtype composition and have only been identified in one individual (McCutchan, 2006). Almost all subtypes and CRFs can be detected in Sub-Saharan Africa. The rest of the world shows a specific geographic distribution pattern for HIV-1 subtypes that can be attributed to the founder effect, the first HIV-1 subtype that are successfully introduced into a population is likely to became the most predominant subtype (Buonaguro *et al*, 2007, Thomson and Najera, 2005). Figure 1.8 shows the global distribution of HIV-1 subtypes.

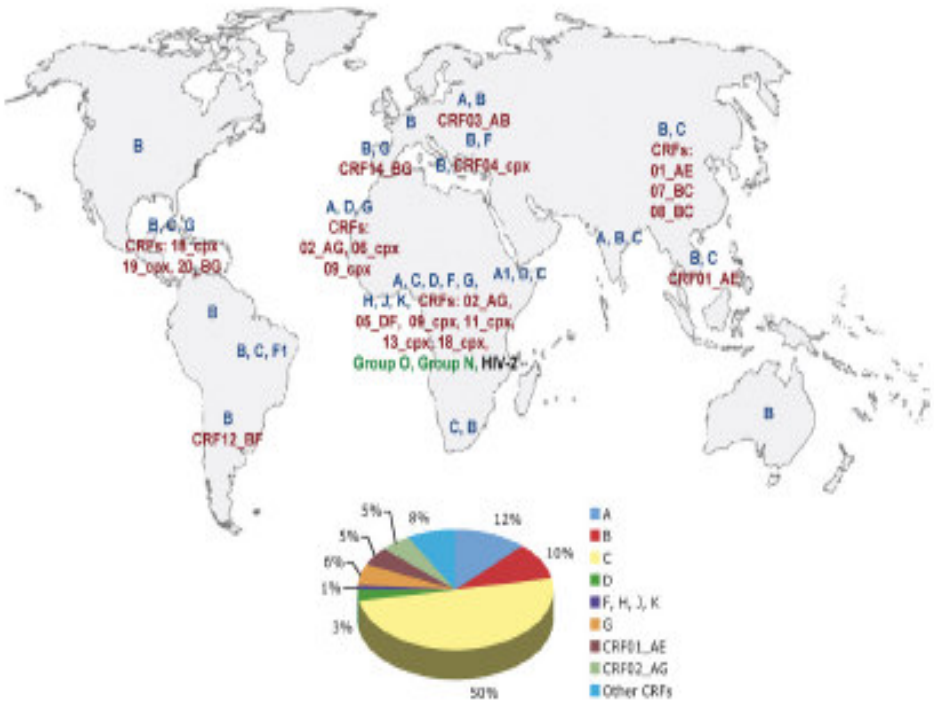

Figure 1.8: Geographic distribution of HIV genetic forms (Ramirez *et al*, 2008)
HIV-1 subtype diversity can have a major impact on antiretroviral treatment and the development of drug resistance. It may affect the genetic barrier, defined as the amount of viral mutations necessary to result in a drug resistant virus as polymorphisms associated with a particular HIV-1 subtype can affect the genetic barrier towards a particular antiretroviral drug. (Beerenwinkel *et al*, 2005).

1.8 Mother-To-Child-Transmission (MTCT)

In the absence of interventions MTCT of HIV-1 occurs at a rate of more than 15%-30% and is one of the major causes of AIDS in children under the age of 15 years. Approximately 370 000 new infections occur in children under the age 15, of which 90% are in Africa (UNAIDS, 2010).

HIV-1 is transmitted from mother-to-infant during pregnancy, labour and delivery or during breastfeeding. It is estimated that 25-48% of transmission in developing countries occurs during labour and delivery, approximately 20% of infections occur during breastfeeding (De Cock *et al*, 2000) and 20-30% in-utero (Farquhar *et al*, 2010). The risk of transmission from mother-to-infant has been shown to decrease with the administration of antiretroviral drugs. In 1994 it was shown that the administration of AZT to pregnant women reduces the risk of MTCT by 70% (Conner *et al*, 1994) and when antiretroviral therapy during pregnancy, throughout labour and therapy to the neonate is combined with caesarean section only 2% of infants become

infected (The International Perinatal HIV Group, 1999). Highly active antiretroviral therapy (HAART) regimens have shown similar results (Cooper *et al*, 2002).

Nevertheless, the cost of these regimens has limited their availability in developing countries. In 1999 a study done in Uganda showed that sd-NVP decreases the risk of MTCT (Guay *et al*, 1999). NVP is a Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI) that inhibits viral replication. Developing countries that have implemented preventative MTCT programs have been able to lower MTCT to below 10% (UNAIDS, 2010). However a problem of using an sd-NVP regimen is the development of HIV-1 resistance mutations to NVP due to the low genetic barrier of HIV for NVP (Ren and Stammers, 2008). Resistance occur in 19-69% of mothers that receive a single dose of NVP. Different HIV-1 subtypes have been shown to developed resistance to a single dose of NVP at different rates (46-69%) with the highest incidence of resistance seen in HIV-1 subtype C (Eshleman *et al*, 2001, Eshleman *et al*, 2005 and Kurle *et al*, 2007).

1.9 Antiretroviral drugs

There are 32 antiretroviral drugs approved by the Federal and Drug Administration (FDA) for treatment of HIV-1 positive patients in 2010 (Federal and Drug Administration, http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm (November 2010)). These include combination of drugs, but exclude generic forms. They are divided into different classes according to their mode of action. The classes available are Nucleoside and Nucleos(t)ide Reverse Transcriptase Inhibitors (NRTI), Non-nucleoside Reverse Trascriptase Inhibitors (NNRTI), Protease Inhibitors (PI), Integrase Inhibitors, Fusion Inhibitors and CCR5 receptor antagonist.

Zidovudine (AZT), an NRTI, was the first antiretroviral drug available for treatment of HIV-1 positive patients that was approved for use by the FDA in 1987 after it was found to be effective in the treatment of AIDS patients (Fischl *et al*, 1987). Currently there are 8 NRTIs approved by the FDA and 4 combinations of NRTI drugs. NRTIs enter the host cell where they are triphosphorylated by the cellulular enzymes Tenofovir is the only NRTI licensed for HIV therapy, taken in its monophosphorylated form (Fung *et al*, 2002). The triphosphorylated synthetic nucleosides and then compete with natural deoxynucleoside triphosphates (dNTPs)

for incorporation into the newly synthesised DNA chains. This incorporation terminates the elongation of the chain and inhibits the viral replication (Shafer, 2004).

Four NNRTIs have been approved by the FDA, Nevirapine (NVP), Efavirenz (EVF) Etravirine (ETR) and Delavirine (DLV). NVP was approved in 1996 by the FDA for use with AZT to obtain prolonged HIV-1 suppression. All the NNRTIs bind to the same area in the RT enzyme and have the same functional properties. The NNRTIs bind to the HIV-1 reverse transcriptase in the hydrophobic pocket located between the β6-β10- β9 and β2-β13-β14 sheets of the p66 subunit. Binding of the NNRTIs to this site displaces the catalytic aspartate residues relative to the polymerase-binding site and inhibits HIV-1 replication (Shafer, 2004).

HIV therapy was limited to the NRTIs until the PIs were FDA approved during 1995. Studies showed that NRTIs in combination with PIs and NNRTIs were more effective in long term suppression of HIV-1 (Deeks et al, 1997, Markowitzet al, 1995). Currently there are 9 PIs approved by the FDA. PIs bind to the active site of the protease enzyme and prevent the cleavage of the Gag and Gag-Pol polyproteins to yield the structural proteins and enzymes of the HIV-1 virus (Shafer, 2004).

The development of resistance has let the development of new antiviral drugs for treatment of resistant viruses. Fusion and integrase inhibitors and a co-receptor antagonist have been approved by the FDA. The integrase inhibitor acts against strand transfer by binding to the target DNA site of the integrase enzyme. The fusion inhibitor, enfuvirtide, corresponds to part of the heptad repeat domain HR2 which consists of residues 127-162 of the gp41, the transmembrane glycoprotein subunit of the envelope glycoproteins. The gp41 subunit promotes fusion of viral and cellular membrane and entry of the viral core into the cell. The heptad repeat domains HR1 and HR2 form a helical bundle that contains trimers of each domain during fusion. Enfuvirtide is a synthetic peptide that inhibits the interaction between HR1 and HR2 by mimicking HR2 (Shafer and Shapiro, 2008). Maraviroc, a CCR5 antagonist, is the only licensed antiretroviral drug that does not target a viral-encoded protein but inhibits HIV-1 replication by blocking a host cell co-receptor. This drug is used in patients, harbouring viruses with CCR5 receptor tropism (Fätkenheuer *et al*, 2005).

The South African National Antiretroviral Treatment guidelines, in 2006 recommended 2 regimens for treatment of adult patients. Regimen 1 consisted of two

NRTIs, Stavudine (d4T) and Lamivudine (3TC), and 1 NNRTI, Nevirapine (NVP) or Efavirenz (EFV). Therapy naïve patients started with regimen 1 and if they failed on the first line regimen their treatment had to be adjusted to regimen 2 that consisted of 2 NRTIs, Zidovudine (AZT) and Didanosine (ddi), and 1 boosted PI, Lapinavir/ritonavir (LPV/r) (Republic of South Africa, 2004). In 2010 this policy chanded to Tenofovir (TDF), 3TC or Emtricitabine (FTC), and EFV, or NVP as first-line regimen followed by zidovudine (AZT), Lamivudine (3TC) and LPV/r as second-line regimen (Republic of South Africa, 2010).

1.10 Drug of interest for the purpose of this study

In the Western Cape Province, AZT and NVP are the drugs used in the prevention of MTCT program. In 2010 the national policy was changed to include this dual regimen for use in the National PMTCT program.

1.10.1 Zidovudine (AZT)

A structural representation is in Figure 1.9. AZT was the first drug approved by the FDA in 1987 after a study done by Fischl *et al* (1987) found that AZT was effective in reducing the mortality and rate of opportunistic infections of HIV-1 positive patients. AZT is an NRTI and inhibits the replication of HIV-1 by acting as a substitute for dNTPs. Chain termination follows after the incorporation of the inhibitor due to the fact that it does not contain a 3'-OH group (Arts *et al*, 1998). After the success of AZT, other NRTIs were developed and initially favoured over AZT due to the adverse side effects and the development of drug resistance mutations to AZT. Today, used in a lower dose, AZT, in combination with other NRTIs and NNRTIs has remained one of the most effective drugs available for treatment of HIV-1 positive patients (Gulick, 1997).

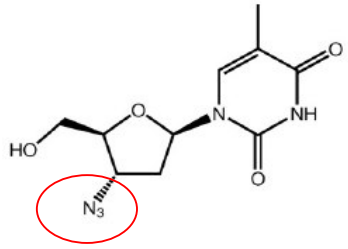

Figure 1.9: Structure of Zidovudine (AZT) with substitution of 3'-OH group with a nitrile group. (Menendez-Arais, 2008).

1.10.2 Nevirapne (NVP)

NVP showed the potential in suppressing HIV-1 replication in initial studies (Davey *et al*, 1993, Merluzzi *et al*, 1990, Saag *et al*, 1993). The NNRTIs bind to the RT enzyme close to the active site and prevent the binding of the enzyme substrate to the active site of the RT, thus inhibiting HIV-1 replication (De Clerq, 2004, Shafer, 2004). Although a potent inhibitor of HIV-1, the development of drug resistant virus strains was seen in all initial studies with NVP as a monotherapy (Saag *et al*, 1993, Davey *et al*, 1993). In studies done where NVP was used in combination therapy, it was found to be very effective and the drug was approved by the FDA in 1996 for use in combination with other antiretroviral therapies. Its use in pediatric patients was approved in 1997 and the effectiveness of the drug in the prevention of MTCT was described in 1999 in Uganda cohort of HIV-1 positive woman and their infants receiving a single dose of NVP (Guay *et al*, 1999). The structure of NVP is presented in Figure 1.10.

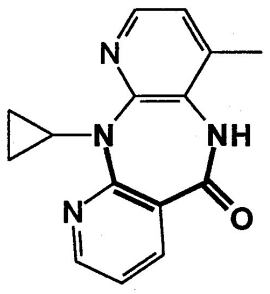

Figure 1.10: Structure of Nevirapine (NVP) (De Clerq, 1999).

1.11 HIV-1 drug resistance

The first HIV-1 antiretroviral drug resistance was described in 1989 by Larder *et al*. when it was noted that patients become less likely to respond to AZT therapy after prolonged treatment.

Drug resistance is the reduction of drug susceptibility of a virus compared to the wild-type virus by the presence of genetic mutations. Drug resistance mutations occur in the *protease* and *reverse transcriptase* genes and can be attributed to the poor proofreading ability of the RT enzyme during the replication cycle that causes genetic variation in HIV-1. The average error rate of the enzyme is 1 error per 2000-5000 nucleotides polymerized (Menéndez-Arias, 2008) and it has been estimated that single

point mutations occurs 10 000 to 100 000 times per day in an untreated HIV-1 positive person. The number of HIV-1 variants is further increased by the extremely high rate of replication of HIV-1, up to 10^{10} viral particles produced per day (Coffin, 1995). This contributes to the high degree of diversity of HIV-1 during the course of an infection.

Single point mutations, deletions and insertions during the HIV-1 replication cycle lead to the development of drug resistance mutations. The size and variation of the HIV-1 population within an individual, the suppression of viral replication during antiretroviral therapy, the path by which a mutation is acquired, the effect that the mutation will have on viral fitness and the influence that the mutation will have on drug susceptibility, can all play a role in the rate and amount of drug resistant mutations that develop (Shafer, 2002).

There is a standard numbering system for drug resistance mutations based on their amino acid positions within the *protease* and *reverse transcriptase* gene. The subtype B consensus sequence is used as the reference sequence. This consensus sequence was formed from the alignment of sequences available in the HIV Sequence Database (Kuiken *et al*, 1999) at the Los Alamos National Laboratory. Mutations are described by using the first letter of the consensus subtype B amino acid, followed by the position of the mutation in the sequence, followed by the letter of the amino acid that indicate the mutation e.g. M184V. When a mixture of more than one amino acid is reported at one position, the different amino acid letters are written after the position separated by a forward slash e.g. M184M/V indicating a mixture of the wild-type amino acid and the mutant amino acid (Shafer, 2004).

Some resistance mutations, can on their own, cause resistance to one or more drugs, e.g. M184V, and are referred to as primary or major resistance mutations. Other mutations only cause resistance if present in combination with major resistance mutations e.g. E138A, and are refered to as secondary or minor mutations (Shafer, 2004).

The accumulation of mutations such as M41L, D67N, K70R, L210W, T215F or T215Y and K219Q or K219E can cause high level of resistance against the NRTIs. These mutations are known as the thymidine analogue mutations (TAMs) as they are found most often when the patients are on a regimen containing AZT and d4T

(Shafer, 2004). These mutations are frequent in low-income countries, like South Africa that use thymidine analogues in the first and second line regimens (Shafer and Schapiro, 2008). There are two patterns seen for the accumulation of TAMs. The type 1 pattern includes mutations M41L, L210W and T215Y and the type 2 pattern includes mutations D67N, K70R, T215F and K219Q/E. The type 1 TAMs cause higher levels of resistance than type 2 TAMs (Shafer and Schapiro, 2008).

HIV-1 diversity also plays a role in the development of resistance mutations. It was found that naturally occurring polymorphisms in non-B HIV-1 subtype can contribute to drug resistance (Cornelissen *et al*, 1997). Polymorphisms in non-B subtype HIV-1 sequences also create shorter pathways for the selection of drug resistance mutations. The pathway for the selection of the V106M mutation, that causes resistance to NNRTIs, is shorter in HIV-1 subtype C than in subtype B viruses. The polymorphism at codon 106 of GTG, found in 94% of HIV-1 subtype C sequences, can be changed to ATG with a single mutation. This polymorphism is rarely seen in the HIV-1 subtype B viruses where the codon is GTA and 2 mutations are needed for the V106M resistance mutation to develop (Brenner *et al*, 2003). The implications of the effect of subtype associated polymorphism on the algorithms used for drug resistance and the treatment options for patients with non-B subtypes HIV-1 still are not fully understood.

1.11.1 Protease inhibitor (PI) resistance

The PR targets the cleavage sites on the Gag and Gag-Pol polyproteins to produce the structural proteins and enzymes of the virus. More than 30 mutations are associated with PI resistance (Johnson *et al*, 2008). Resistance to PI's develops due to structural changes that reduce the binding of the protease to the inhibitor.

1.11.2 Nucleoside analogue Reverse Transciptase (NRTI) resistance

The mechanism of NRTIs is to block the elongation of the proviral DNA during reverse transcription and terminate the DNA chain formation. A drug enters the host cell where it is phosphorylated and then competes with natural dNTPs for incorporation into the newly synthesised DNA chains. This incorporation terminates the elongation of the chain and inhibits the viral replication. Resistance to NRTI's develops via two biochemical processes. One method is to decrease the incorporation of NRTI's by the action of mutations that allow RT to discriminate between NRTI's

and natural dNTPs during phosphorylation and thus prevent their incorporation into the newly formed DNA chain. Such discriminatory mutations are the M184V, Q151M, K65R and L74V. The second process is phosphorolytic removal of the incorporated NRTI's from the newly formed chain to allow continued polymerisation and strand elongation. Mutations that facilitate phosphorylitic removal of incorporarated NRTIs are the TAMs, the T69 insertion and accessory mutations (Shafer, 2004, Shafer and Schapiro, 2008).

1.11.3 Non nucleoside analogue Reverse Transciptase (NNRTI) resistance

The NNRTIs bind to the HIV-1 reverse transcriptase in the hydrophobic pocket located between the β6-β10- β9 and β2-β13-β14 sheets of the p66 subunit. Binding of the NNRTIs to this site displaces the catalytic aspartate residues relative to the polymerase-binding site and inhibits HIV-1 replication. Resistance arises with the development of mutations in or next to the NNRTI binding site. Only one or two mutations in this area are necessary for the development of high level resistance, therefore HIV has a low genetic barrier to the development of NNRTI resistance. These mutations often lead to a high level of cross resistance between the different NNRTI's. Resistance occurs due to mutations causing a reduction in the binding potential of the NNRTI's (Y181C and Y188C/I/H), stabilizing of the RT conformation (K103N) or reduction of protein inhibitor contact (L100I, V106A and V108I) and charge repulsion (K101E, E138K) (Ren and Stammers, 2008).

Mutations for NNRTI resistance can be categorized into 4 groups: 1) major NNRTI resistance mutations that cause resistance to one or more NNRTI's and that develop early, 2) minor NNRTI resistance mutations that occur in combination with major NNRTI resistance mutations, 3) minor non-polymorphic mutations that occur alone or in combination with other mutations that cause low-level NNRTI resistance and 4) polymorphic mutations that influence the effect of other NNRTI resistance mutations (Shafer and Schapiro, 2008).

Mutations in the envelope gene and the *integrase* gene that confers resistance to the entry inhibitors and the integrase inhibitors have been identified but will not be discussed as the inhibitors are not feely available in South Africa and have no bearing on the project.

1.12 Drug resistance testing

Different methods are available for detection of drug resistance mutations. The most commonly used method is genotypic testing which uses gene sequencing to detect mutations that could result in decreased susceptibility to a drug. Phenotypic testing, which measures the amount of resistance that the viral population shows in an *in vitro* replication system, is more expensive and time consuming, although one advantage is that it can be used to study complex interactions of mutations (Shafer, 2004, and Hanna and D'Aquila, 2001).

Genotypic testing relies on population based sequencing and reports consensus sequence at each nucleotide. It can detect mutations in mixed populations that are too low to cause drug resistance on its own. This could provide insight in the potential for resistance to emerge and it can also detect transitional mutations that do not cause drug resistance by themselves but indicate the presence of selective drug pressure. Another advantage of genotypic assays is that they have a more rapid turn-around than phenotypic tests and are less costly.

The presence of some resistance mutations can lead to the phenotypic sensitivity to other drugs for example, the lamivudine-selected M184V mutation can lead to the reversal of AZT resistance in the presence of several nucleoside analog-resistance mutations (NAM's) (Tisdale *et al*, 1993).

Detection of significant minority species also remains a problem with sequence-based methods. Most genotypic resistance assays can detect minority resistance mutations at approximately 20%. In patients heavily treated and have since change a level of therapy due to resistance, strains containing the resistance mutations will become undetectable within weeks as the viral population revert back to a predominant 'wild type' strain without resistance mutations. The resistant virus will however reappear as soon as the drug is reintroduced (Devereux *et al*, 1999).

Studies have shown that genotypic testing is more useful clinically than phenotypic testing (Baxter *et al*, 2000, Clingolani *et al*, 2002, Cohen *et al*, 2002, Durant *et al*, 1999, Haubrich *et al*, 2001, Tural *et al*, 2002).

Commercial assays for genotyping, like the ViroSeq™ HIV-1 genotyping assay and the Trugene™ HIV-1 genotyping assay, are FDA approved and available. The high costs prohibit the use in recourse-limited settings such as the South African public

programme. Also, the sequencing primers, developed for industrialised countries, where HIV-1 Subtype B is the prevalent subtype, do not detect non-subtype B viruses as effectively. (Beddows 2003, Jagodzinski 2003)

New sensitive assays are available for resistance testing, including single-genome sequencing (Palmer *et al*, 2005), allele-specific Polymerase Chain Reaction (PCR) (Bergroth *et al*, 2005, Menzner *et al*, 2003, and 2005), hybridisation assays (Flys *et al*, 2005) phenotypic analysis using *S. cerevisiae* (Nissey *et al*, 2005), massively parallel sequencing in microfrabricated PicoTiterPlates (Leamon *et al*, 2003), pyrosequencing (O'Meara *et al*, 2000) and parallel allele-specific PCR (Cai and Gochin, 2007). These assays are very specialised, generally costly, and not adapted for use in diagnostic monitoring of patients on antiretroviral treatment.

1.13 Optimisation of the Polymerase Chain Reaction (PCR)

The aim of this study is to optimize and validate a genotyping assay. In this section the PCR method will be discussed in relation to optimisation.

1.13.1 Polymerase Chain Reaction (PCR)

The PCR method was developed in 1985 by Kary B. Mullis (Mullis and Faloona, 1987). The basic PCR method involves DNA heat denaturing, primer annealing and DNA extension in repeated cycle steps (Saiki *et al*, 1988). The standard conditions will amplify most target sequences but new PCR protocols require optimisation to perform efficiently in repetitive conditions. The following factors must be considered in optimisation of a PCR, the enzyme, dNTP and magnesium concentrations, template quality and input, and additives or enhancers for PCR. Primer design, annealing temperature and extension times also is important in PCR optimisation (Innis *et al*, 1990, Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010).

1.13.2 Enzyme concentration

If the enzyme concentration is too high, non-specific background products may be seen and if the concentration is too low and insufficient amount of product is amplified (Innis *et al*, 1990, Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010).

1.13.3 Deoxynucleotide triphosphates (dNTP) concentration

The four dNTP's should be used at equivalent concentration to minimize misincorporation errors. The specificity and the fidelity of PCR are increased by using lower dNTP concentration. Low dNTP concentrations minimize the mispriming at non-specific sites and reduce the likelihood of extending misincorporated nucleotides. The recommended concentration for dNTP's is 200µM of each dNTP. If the concentration is increased, the magnesium concentration must also be increased as an increase in dNTP's reduces the free Mg^{2+} and interfers with polymerase activity and primer annealing (Innis *et al*, 1990).

1.13.4 Magnesium concentration

The magnesium concentration can affect the primer annealing, denaturing temperatures of the template and PCR product, product specificity, formation of primer-dimers, and enzyme activity and fidelity. The magnesium concentration must be 0.5-2.5mM for a PCR. Lower magnesium concentrations increase the specificity of priming and amplification and higher concentrations lead to non-specific amplification. DNA template concentrations, chelating agents like EDTA, dNTP concentration and the presence of proteins can affect the amount of magnesium in a reaction (Innis *et al*, 1990, Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010).

1.13.5 Template quality and amount of input

The quality and purity of the template are crucial for it success. The amount of template influences the performance of the PCR. Low amount of template will require specific modifications to the PCR, such as an increase in cycle number, redesign of primers, use of specific enzymes to accommodate input volume. Large amounts of template can lead to inhibition of the PCR. (Ausubel *et al*, 2006, Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010).

1.13.6 Additives and enhancers for PCR

Some components enhance the efficiency, specificity or yield of a PCR. These components can sometimes also cause inhibition, therefore they should be used with caution so care should be taken when using them. Nonionic detergents (Triton X-100) neutralizes charges of ionic detergents used in the extraction methods. Enzyme-stabilising proteins (bovine serum albumin), enzyme-stabilising solutes (Betadine) and enzyme-stabilising solvents (glycerol) and solubility-enhancing solvents (Dimethyl sulfoxide) will achieve higher yields of PCR product by stabilizing or enhancing the polymerase enzyme (Ausubel *et al*, 2006, Promega, Protocol and Applications guide http://www.promega.com/ paguide/chap1.htm , November 2010). Reducing agents e.g. dithiotheritol (DTT), reduce disulfide bonds and provide free Mg^{++} for the reverse transcriptase reaction (Nagai *et al,* 1998, Ralser *et al*, 2006).

1.13.7 Primer design, annealing temperature and extension times

An optimal primer must hybridize effectively to the target template with no or little hybridization to other DNA templates present. The primers must be designed to be specific for the target template. Primers are usually designed to be exactly complementary to the template. Some primers have mismatches and the closer the mismatches are to the 3' end of the primer, the higher the chance that it will not amplify. A primer should be 20- 30 bases in length. Sufficient primer length could help with specific amplification of the target sequence. The primer sequence must be designed to have the same GC content than the template and it should be between 40-60%. There should not be three G or C bases near the 3' end of the primer to minimize non-specific annealing. Avoid primers with unusual sequence distributions such as long areas of polypuridines, since this can form secondary structures. To avoid primer-dimer formation, primers that are not complementary to one another must be designed. Primer-dimers are observed when the 3' end of the one primer bind to the 3' end of the other and DNA polymerase extend each of the primer ends. The melting temperature (T_m), the temperature at which 50% of the primer molecules are annealed to the template, of the primers used in the PCR must be within 5°C of one another so that the efficiency of the primers is at the same temperature. There are several computer programs e.g. Primerplex, Primer 3, OligoPerfect™ Designer, that can help in primer design. The MyBio webpage contain links to many primer design tools (http://mybio.wikia.com/wiki/Primer_design_tools) (Ausubel *et al*, 2006, Innis

et al, 1990, Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010).

1.13.8 Primer selection for sequencing

Fluorescence-based cycle sequencing reactions are used to sequence DNA templates from PCR products. A single primer is used for each PCR reaction and primer extension is terminated at different sequence lengths with the size of the primer corresponding to the sequence length and the particular fluorescent label corresponding to the terminal base. The quality of sequencing results will depend on the quality of the sequencing primer. The following criteria must be met for sequence primer design: the primer sequence must be unique to the region of interest; the primers must not contain hairpin loops; the melting temperature of the primer must be appropriate for cycle sequencing and the primer must be pure (Li *et al*, 2008).

The size of the sequence obtained with one primer reaction is approximately 500 bp. If a larger DNA region must be sequenced the region must be broken down into smaller fragment for sequencing. This is done by obtaining sequence data for the DNA fragment that must be sequenced. Primers are designed to correspond to shorter sequences of the region of DNA of interest (Strauss *et al*, 1986). These shorter fragments can be assembled using software like the Sequencer software V4.7 (Gene Codes Corporation, Ann Arbor USA) to obtain a complete sequence of the region of interest.

1.14 Validation of methods

Validation is the evaluation of a process to determine the fitness of the process for a particular use. There are different types and levels of validation. A full validation is performed when the method is developed and implemented for the first time. A partial validation is performed when the method was already validated and modifications are made to it. This validation can range from intra-assay accuracy and precision determination to a nearly full validation. A cross-validation is where the original validated method serves as a reference and the revised method is compared to it. In this study partial and cross validation methods will be applied (US Department of Health and Human Services, 2001).

To validate a method, quality control (QC) samples are used to evaluate the performance of the method and comparison between methods is done using the same

samples. Acceptability of the performance of a method is determined by the accuracy, precision, specificity, sensitivity, reproducibility and stability of the method. Accuracy describes the closeness of the method result to the true result of the assay. Precision describes the closeness of individual results to one another if repeat measurements are made. Specificity determines the ability of the method to detect the correct analyte and sensitivity determines the ability of the method to detect the analyte. Reproducibility determines the ability of the assay to obtain the same result after repeated testing of the same sample. Stability determines the stability of the analyte in the sample for testing. For the method to be valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision (Jacobson and Romatowski, 1996, US Department of Health and Human Services, 2001).

1.15 Aim of the study

The first aim of this study is to validate an in-house drug resistance assay, adapted from Jacobs *et al* (2008) as a diagnostic and research tool. The following parameters will be changed (a) input RNA volume into the PCR reaction, (b) the denaturing of RNA and (c) primer and magnesium concentrations. The output will be compared withexternal quality control samples. A second aim is to study the development of antiretroviral resistance in patients on the Western Cape MTCT regimen. This is done by testing patient samples with the newly optimised and validated genotyping assay. A third objective is to improve the optimised assay to be more cost effective and less technical and to validate it as a routine diagnostic assay. This is done by investigating the primer selection for both the PCR and sequencing reactions, adding an additive to the PCR reaction mix, comparing annealing temperature of the primers, volume reduction of the second round PCR reaction mix, and using sequencing buffer to reduced cost of the sequencing reaction.

Chapter 2

Chapter 2

2. The optimization and validation of a Two Fragment In-House HIV-1 antiretroviral resistance assay

2.1 Introduction

The aim was to optimise and validate an in-house genotypic drug resistance assay published by Jacobs *et al* (2008) for a research study. The method by Jacobs *et al* (2008) was adapted from a method by Plantier *et al* (2005). The same primers were used in the PCR reaction for both assays with different sequencing primers. The optimized assay is referred to as "Two Fragment In-house assay (TFIH)". To optimize the assay by Jacobs *et al* (2008) the following parameters were investigated: the input volume of RNA into the PCR reaction, the inclusion of a RNA denaturing step prior to reverse transcription and optimal primer and magnesium concentrations. Following optimisation, the assay was validated by comparison with the ViroSeq™ HIV-1 Genotyping Assay, using external quality control (QC) samples. After optimization and validation, the TFIH assay was used to study the prevalence of genotypic-antiretroviral resistance in patients treated according to the Western Cape Provincial PMTCT protocol.

2.2 Methods

Table 2.1 is a comparison between the Jacobs *et al* (2008) assay, described in Appendix A, and the TFIH assay.

2.2.1 Samples

Six isolates were received from Lynn Morris (National Institute Communicable Diseases (NICD) AIDS Virus Research Unit). The sequences for 3 of the samples DR10, DR19, and DR28 were published in Pillay *et al* (2008). Genebank accession number AY043173, AY589880, and AY589885. These samples were used for sensitivity determination of the TFIH assay. Samples NVP8b, NVP64 and NVP186 were not published before. The expected resistance mutations as supplied by Lynn Morris, are listed in Appendix B. Six samples were received from the Round Robin HIV-1 genotyping assessment system, numbered WQ1 to WQ6, from the University of Würzburg, Department of Virology (Bodem, 2007). The expected resistance

mutations are in listed in Appendix C. These samples were used in the validation of the TFIH assay.

Table 2.1: A comparison of the method used in Jacobs *et al* 2008 and the TFIH assay

Steps in genotyping assay	**Jacobs et al (2008)**	**TFIH**
Plasma used for extraction	1ml	1ml
RNA extraction	QIAamp UltraSens Kit	QIAamp UltraSens Kit
First round PCR		
*Denature of RNA	No RNA denaturing	RNA denatured
*Input volume	5µl	10µl
Additives	None	None
Number of primers used	4	4
Second round PCR		
*Input volume	5µl	2µl
Reaction volume	50µl	50µl
Number of primers used	4	4
Annealing temperature	55ºC	55ºC
Sequencing reactions		
Volume of BigDye™ Terminator v3.1 ready reaction mix	4µl	4µl
Sequencing primers	8	8
Annealing temperatures	45ºC and 50ºC	45ºC and 50ºC

* Differences between assays

2.2.2 Materials and Methods

2.2.2.1 Sample preparation

Frozen isolates were received from the NICD AIDS Virus Research Unit and frozen plasma samples were received from the Round Robin HIV-1 genotyping assessment system and stored at -70ºC until use. RNA was extracted and the HIV-1 RNA loads of the extracted isolates were determined, in order to make appropriate serial dilutions of the viral isolates for the assay validation. The extracted RNA of these isolates and the Round Robin samples were subsequently used to optimise the PCR and sequencing reactions and to validate the TFIH assay.

2.2.2.2 RNA Extraction

The QIAamp UltraSens Kit (QIAGEN GmbH, Germany) was used for RNA extraction from 1ml plasma. This method utilises spin column technology with the selective binding properties of a silica-gel-membrane that was first described by Vogelstein and Gillespie (1979). Samples were added to a lyses buffer and placed in a spin column. The silica adsorbs the RNA at specific salt and pH concentrations while contaminants are washed from the membrane. The pure RNA was then eluted from the membrane with Tris buffer or water. The manufacturer's instructions were followed and the RNA was eluted in two elution steps with 30 µl of AVE buffer added each time to obtain a total elution volume of 60 µl.

2.2.2.3 HIV-1 viral load assay

The NucliSens EasQ® system V1.2 (bioMérieux, Boxtel, Netherlands) with 1ml plasma sample input was used according to the manufacturer's instructions to determine the viral load.

2.2.2.4 ViroSeq™ HIV-1 Genotyping assay

The ViroSeq™ Genotyping system V2.0. (Celera Diagnostics, CA, USA) was used as a reference assay and performed according to the manufactures instructions, excluding the extraction method supplied by the manufacture.

2.2.2.5 Enrolment in the Quality Control of Molecular Diagnostic (QCMD) assessment system

Quality Control for Molecular Diagnostics (QCMD) is a European quality assurance program established to advancing the quality of molecular diagnostics through External Quality Assessment (EQA), Proficiency Testing (PT) and other quality initiatives (Pandit *et al*, 2008).

2.2.2.6 Polymerase Chain Reaction (PCR)

This method described below was used for all PCR reactions performed, unless otherwise stated. The Access RT-PCR System (Promega, Madison, USA) was used for the first round reverse transcriptase-PCR (RT-PCR) reactions in a one step protocol. The PCR system that was used employs the Avian myeloblastosis virus Reverse transcriptase (AMV RT) and *Thermus Flavus* (*Tfl*) DNA polymerase to transcribe RNA to cDNA and amplify cDNA (Abramovici, 2001). The PCR primers are listed in Table 2.2 (Plantier *et al*, 2005). The first round reaction mix that consisted of AMV/T*fl* 5X reaction buffer at a 1X concentration, a dNTP-mix at 0.2mM each, primers at 40pmol, 2mM $MgSO_2$, AMV RT 0.1U/µl, T*fl* DNA Polymerase 0.1U/µl and nuclease-free water up to a final volume of 50µl. PCR cycling was done using 9700 PCR thermocyclers (Applied Biosystems, Foster City, USA) at 9600 ramp speed. The reaction mixtures were prepared in 0.2ml PCR tubes (QSP, Porex BioProducts Inc, California, USA). The following cycling conditions applied: First round PCR: For complementary DNA (cDNA) synthesis reaction tubes were incubated at 48°C for 45 minutes to allow for reverse transcription yielding double stranded-DNA (ds-DNA) and then at 94°C for 2 minutes to inactivate AMV Reverse transcriptase and to denature ds-DNA. For exponential amplification of DNA the cycling conditions were: 40 cycles of 94°C for 20 seconds to denature DNA, 55°C for 30 seconds for primer annealing and 68°C for 90 seconds for primer extension. This is followed by a final 5 minutes at 68°C to complete primer extension. The product was then stored at 4°C until the second round reactions could be done. The *Taq* DNA Polymerase in storage buffer B system (Promega, Madison, USA) was used for the second round PCR reaction mixture using the first round RT-PCR product as template input. The thermostable *Thermus aquaticus* (*Taq*) DNA polymerase was used to amplify the PCR product from the first round PCR. The PCR primers used were listed in Table 2.2 (Plantier *et al,* 2005). Five µl of the first round RT-PCR

reaction product was added to the second round reaction mix which consisted of 5X storage buffer B at a 1X concentration, PCR Nucleotide mix (Promega, Madison, USA) at 0.2mM each, primers at 40pmol each, 1.5mM $MgCl_2$, Taq DNA Polymerase at 2.5U/µl, and nuclease-free water up to a final volume of 50µl. The cycling conditions for the second round PCR were: One cycle at 94°C for 2 minutes for DNA denaturing followed by 40 cycles of 94°C for 20 seconds for DNA denaturing, 55°C for 30 seconds for primers annealing and 68°C for 90 seconds for primer extension. This was followed by a final extension for 5 minutes at 68°C. The product was then stored at 4°C until it could be visualized.

Table 2.2: Primers used for first and second round PCR of the TFIH assay

Protease Primer	**HBX2 number**	**Primer Tm**	**Primer direction**	**Primer Sequence**
outer 3'prot1	2703-2734	57.6°C	*Reverse*	5'- GCA AAT ACT GGA GTA TTG TAT GGA TTT TCA GG –3'
outer 5'prot1	2082-2109	57.0°C	*Forward*	5'- TAA TTT TTT AGG GAA GAT CTG GCC TTC C -3'
inner 3'prot2	2621-2650	55.8°C	*Reverse*	5' AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC –3'
inner 5'prot2	2136-2163	68.2°C	*Forward*	5'- TCA GAG CAG ACC AGA GCC AAC AGC CCC A –3'
Reverse transcriptase Primer				
outer Mj4	3399-3420	54.8°C	*Reverse*	5'- CTG TTA GTG CTT TGG TTC CTC T –3'
outer Mj3	2480-2499	54.3°C	*Forward*	5- AGT AGG ACC TAC ACC TGT CA –3'
inner NE13	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3
inner A35	2530-2564	58.4°C	*Forward*	5'- TTG GTT GCA CTT TAA TTC CCA TTA GTC CTA TT –3'

2.2.2.7 Visualization of the PCR product with agarose gel electrophoresis

The PCR product was identified according to product size (514bp for the protease gene and 804bp for the reverse transcriptase gene) with gel electrophoresis on a 0.8% agarose gel prepared with LE analytical grade agarose (Whitehead Scientific, Cape Town, RSA) in 1 x TAE Buffer (0.04M Tris acetate, 0.001M EDTA). Five µl of 5mg ethidium bromide (Promega, Madison, USA) was added to the gel to intercalate ds-DNA. Two µl loading buffer (Promega, Madison, USA) was mixed with 5µl PCR product. Each specimen was sequentially loaded in a slot on the gel, including the non template control (a PCR reaction performed with nuclease free water instead of specimen input), positive controls and 1 kilobasepair marker (Promega, Madison,

USA). The electrical current was set at 50 mAmperes for a 50ml gel apparatus. When the products have migrated from the negative to the positive anode sufficiently, as indicated by the migration of the three coloured dyes in the loading buffer, the current was removed and the gel was visualised under ultra violet light at a wavelength of 302nm. At this wavelength ethidium bromide intercalated DNA (PCR products) fluoresces brightly. The gel was photographed using the Syngene™ GeneGenius, version 4.00, program (Synoptics Ldt, Cambridge, United Kingdom).

2.2.2.8 PCR product purification.

The QIAquick Spin Kit (QIAGEN GmbH, Germany) was used to purify the PCR product from the second round PCR for sequencing reactions according to the manufacturer's instructions. The purified PCR product was stored at -20ºC until use.

2.2.2.9 Quantification of DNA with the NanoDrop

The DNA product was quantified with the NanoDrop ND-1000. The NanoDrop ND-1000 is a full-spectrum (220-750nm) spectrophotometer that measures the DNA concentration in 1µl samples with high accuracy and reproducibility. The patented sample retention technology uses surface tension alone to hold the sample in place. The ND-1000 can measure highly concentrated samples without dilution. One µl of sample was measured at wavelengths of 260nm and 280nm. The absorbance at 260nm measures the DNA concentration and the ratio of the sample absorbance at 260nm to 280nm measures the purity of the sample. A ratio of 1.8 to 1.9 indicates highly purified preparations of DNA. The concentration of DNA obtained was used to work out a dilution factor to obtain 10ng-40ng of DNA to use in the sequencing reactions (Ausubel *et al*, 200, Sambrook *et al,* 1989).

2.2.2.10 Sequence of PCR product

Fluorescence-based cycle sequencing reactions was used to sequence DNA templates from PCR products. The ABI Prism® BigDye™ Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Warrington, UK) was used for the sequencing reactions. This kit is based on the dideoxy method developed by Sanger *et al* (1977, 1980). A DNA polymerase is used to synthesise a complementary strand of DNA by elongation of DNA at the 3' end of a primer. The dNTPs are the substrates for DNA polymerisation catalysed by the DNA polymerase enzyme. New bases complimentary to the template strand are incorporated at the 3' end of the elongating primer chain.

The reaction mix contains a mixture of normal dNTPs and substituting 2',3'-dideoxynucleoside triphosphates (ddNTP) which lead to chain termination and are labelled with fluorescent markers (different fluorescent labels are used for each base (A, C, T, G)). A single primer is used for each PCR reaction. Primer extension is terminated at different sequence lengths with the size of the extended primer corresponding to the sequence length and the particular fluorescent label corresponding to the terminal base. Gel-electrophoresis takes place in a long capillary tube that allowing discrimination of products that differ by one base pair. The sequence is determined by detecting the different terminal nucleotides at different wavelengths with laser excitation at the end of a gel. A template quantity of less than 40ng was used to sequence the product. The primers used in the sequencing reactions are listed in Table 2.3.

Table 2.3: Primers used for sequencing of the PR and RT regions with the TFIH assay

PR Primers	**HBX2 number**	**Primer Tm**	**Primer direction**	**Primer Sequence**	**References**
R2051	2486-2504	49.5°C	*Reverse*	5'- TAT RTT GAC AGG TGT AGG T -3'	Personal Communication, John Hackett
pol1D	2251-2271	56.3°C	*Forward*	5'- TCC CTC AAA TCA CTC TTT GGC -3'	Loxton, 2004
JA217	2622-2646	49.5°C	*Reverse*	5'- CTT TTA TTT TTT CTT CTG TCA ATG G -3'	Lindström and Albert, 2003
RT Primers					
pol3D	2869-2884	48.7°C	*Forward*	5' - CAG TAC TGG ATG TGG G - 3'	Loxton, 2004
pol3rev	2882-2901	51.1°C	*Reverse*	5' - CTG AAA AAT ATG CAT CCC CC - 3'	Personal Communication, Susan Engelbrecht
ABB20-3F	2980-2999	51.6°C	*Forward*	5' - ATC AGT ACA ATG TGC TTC CA - 3'	Personal Communication, John Hackett
AK12	2947-2969	50.6°C	*Reverse*	5' - TGG TGT YTC ATT RTT TRY ACT AG - 3'	Lindström and Albert, 2003
AK11	2571-2592	48.4°C	*Forward*	5' - GTA CCA GTA AAA TTA AAR CCA G - 3'	Lindström and Albert, 2003

Cycle sequencing reactions were prepared in a 96 well plate by adding 4µl of BigDye™ Terminator v3.1 ready reaction mix to 1µl of template, 1µl of 5pmol primer and 4µl nuclease free water to obtain a final volume of 10µl. The reaction mix was then exposed to thermocycling conditions repeated 25 times with DNA denaturing at 96°C for 10 seconds and primer annealing for 5 seconds at the sequence primer specific annealing temperature (45°C or 50°C). This was followed by a final product extension at 60°C for 4 minutes. After completion of the cycling, the reactions were sent to the DNA sequencing facility of Stellenbosch University for sequencing on the

ABI 3100 Sequencer (Applied Biosystems, Foster City, USA). The sequencing data from the analyzer was converted to electopherograms by the DNA sequencing facility using the DNA Sequencing Analysis software™, version 3.3 (Applied Biosystems, Foster City, USA).

2.2.2.11 Assembly and analysis of sequences

2.2.2.11.1 Sequence assembly

Sequence electropherograms was analysed, assembled, and edited using the Sequencer software V4.7 (Gene Codes Corporation, Ann Arbor USA). Primers for both the negative and the positive strand are used in the sequencing reaction. This ensures the quality of the sequence being analyzed (Murphy *et al*, 2005).

2.2.2.11.2 Analysis for drug resistance mutations

The fasta format of the sequence was exported and analyzed for resistance mutations with the Stanford HIVdb program for Genotyping resistance interpretation program http://hivdb.stanford.edu/index.html, Updated January 2007 (November 2010). The sample sequence was compared to a consensus sequence of HIV-1 subtype B *pol* sequences for notification and identification of resistance mutations.

2.2.2.11.3 HIV-1 subtyping

The Rega HIV-1 subtyping tool Version 2.0, was used to determine the HIV-1 subtype (http://dbpartners.stanford.edu/RegaSubtyping/) (November 2010) with the jpHMM (jumping profile Hidden Markov Model) (http://jphmm.gobics.de/) (November 2010). The Rega HIV-1 subtyping tool Version 2.0, is designed to use phylogenetic methods to identify the subtype of a specific sequence. The sequence is analysed for recombination using bootscanning methods. The jpHMM compare nucleic acid sequences to multiple alignments of a sequence family for which a classification in subclasses is available (Zhang *et al*, 2006).

2.2.3 Specificity determination

The sequences were analysed by the Basic local alignment search tool (BLAST) of the National Centre of Biotechnology information (http://www.ncbi.nlm.nih.gov) (November 2010) to determine the specificity of the primers (Altschul *et al*, 1990). BLAST finds regions of local similarity between sequences. It compares nucleotide sequences to sequence databases and calculates the statistical significance of matches.

2.2.4 Statistical analysis

A probit regression model in R (version 1.11.1), was used for statistical analysis of the sensitivity of the TFIH assay. It is a freely available environment for statistical analysis and graphics (www.r-project.org) (November 2010).

2.2.5 Optimization

2.2.5.1 Template input volume changes in PCR reactions.

The amount of template was increased to raise the sensitivity of the PCR reaction. Too low a template input in the first round PCR will lead to low amplification rate of RNA. On the other hand it must also be taken into consideration that template overload could lead to inhibition of the PCR, which is especially relevant in the second-round (nested) PCR (Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010, Ausubel *et al*, 2006). The input volume of the RNA template was increased from 5 µl to 10µl of RNA, as described in other studies (Duran *et al*, 2007, Plantier *et al*, 2005) for the first round reaction mix. This would increase the amount of template available for the first round cDNA. The input of PCR product into the second round reaction mix was decreased to 2µl from 5 µl of first round PCR product to prevent inhibition of the PCR reaction through a too high an input of PCR product. The procedure as described in section 2.2.2.4 was followed for amplification. Samples from the NICD AIDS Virus Research Unit were used for this evaluation.

2.2.5.2 Denaturing of RNA before starting the PCR reaction

To eliminate termination sites that may be caused by secondary structures within the RNA, we investigated the effect of RNA denaturing prior to RT-PCR amplification. Ten µl of RNA was placed in 0.2ml thin wall PCR tubes (QSP, Porex BioProducts Inc., California, USA) and incubated for 30 seconds at 65°C and cooled to 48°C for 5 minutes. Alternatively 10µl of RNA was placed in 0.2ml thin wall PCR tubes and incubated for 5 minutes at 70°C and then placed on ice for 5 minutes to snap freeze it. Forty µl of prepared first round PCR reaction mix was added and the procedure described in section 2.2.2.4 was followed for amplification. As a comparison, control specimens, compaired non-denatured RNA were amplified in parallel using the same

protocol (described in section 2.2.2.4 with changes made as in section 2.2.5.1). The same RNA specimens and input volumes were used for all 3 methods compared.

2.2.5.3 Titration of magnesium and primer for the first and second round PCR concentrations

A checkerboard titration was performed to determine optimal primer and Mg^{+} concentrations. Checkerboard titrations using 40pmol, 80pmol and 120pmol of the primers per reaction and concentrations of 1.5mM, 2mM and 2.5mM of magnesium sulphate were prepared for second round PCR reactions. The same procedure as described in section 2.2.2.4 was followed with noted in sections 2.2.5.1 and 2.2.5.2.

2.2.6 Validation

The final protocol for the TFIH assay as described in Appendix D was used for validation. The TFIH as was validated to evaluate the fitness of the assay for use.

2.2.6.1 Validation criteria for this study

2.2.6.1.1 Method of validation

The partial and cross validation methods will be applied in this study since the method being validated will be compared to a previously validated method.

2.2.6.1.2 Samples used for validation

The validation of the TFIH assay was done using samples received from the NICD AIDS Virus Research Unit, for sensitivity testing to determine the limit of detection of the assay, and the Round Robin HIV-1 genotyping assessment system for comparison with the ViroSeq™ HIV-1 Genotyping assay as a reference assay. The TFIH assay was also enrolled in the QCMD assessment system. These samples are discussed in section 2.2.1.

2.2.6.1.3 Reference method

The ViroSeq™ HIV-1 Genotyping system V2.0 (Celera Diagnostics, CA, USA) will be used as a reference assay.

2.2.6.1.4 Acceptance criteria for the method

The same results must be obtained for the QC samples with both methods. The sensitivity and specificity of the investigated assay must not be inferior to the reference (that is at least within the same range as the reference assay).

2.2.6.2 Sensitivity

The 6 viral isolate samples obtained from the NICD AIDS Virus Research Unit were used to determine the analytical sensitivity (limit of detection) of the TFIH assay. The viral load of the isolates were determined with the NucliSense Easy Q HIV-1 system V1.2 (Biomérieux bv, Boxtel). Ten fold dilutions form 100 000 to 1 copies/ml were prepared. In order to determine the limit of detection we used a probit regression model in R (version 1.11.1).

2.2.6.3 Specificity

To verify the specificity of this in-house reaction, the *pol* gene sequences, obtained, were submitted to blastn algoritm of the National Centre of Biotechnology information (http://www.ncbi.nlm.nih.gov).

2.2.6.4 Comparison of samples from the NICD AIDS Virus Research Unit

To determine if the TFIH assay could detect the resistance mutations in the samples received from the NICD AIDS Virus Research Unit, these samples were tested and analysed for resistance mutations with the Stanford HIVdb program for Genotyping resistance interpretation program http://hivdb.stanford.edu/index.html, Updated January 2007 (November 2010).

2.2.6.5 Comparison of TFIH assay with the ViroSeq™ HIV-1 Genotyping assay

To test if the TFIH assay performs as well as the ViroSeq™ HIV-1 Genotyping assay, 6 QC samples received from the Round Robin HIV-1 genotyping assessment system, described in section 2.2.1, were tested in parallel with the ViroSeq™ HIV-1 Genotyping assay.

2.2.6.6 Quality Control of Molecular Diagnostic system (QCMD)

The TFIH assay was enrolled in the QCMD external quality assessment system for evaluation in 2007.

2.3 Results

This section describes the results obtained from optimisation and validation of the TFIH assay.

2.3.1 Template input volume changes in PCR reactions.

A PCR was performed with a 10µl input volume in the first round PCR and a 2µl input volume in the second round PCR and compared with a PCR with the input volumes in section 2.2.2.4. Figure 2.1 show the results obtained on a 0.8% agarose gel after electrophoresis. Two samples (DR10 and DR19) from the NICD AIDS Virus Research Unit and an HIV-1 negative plasma sample used as a negative control (Neg) were co-amplified. The best amplification was seen with the increased input in the first round PCR and the decreased input volume in the second round.

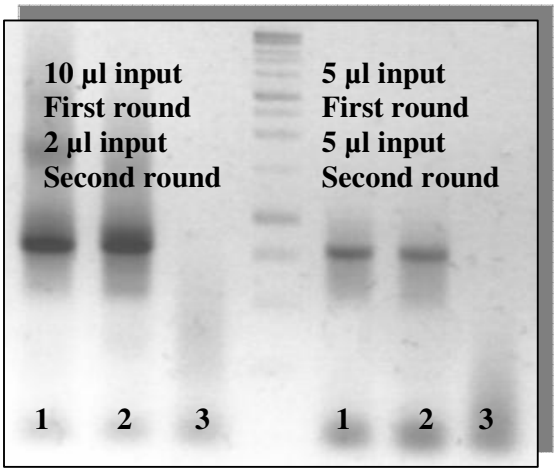

Figure 2.1: DNA agarose gel electrophoresis of the RT fragment comparing a 10µl vs. 5µl input in first round PCR and a decrease to 2µl vs. 5µl input in second round PCR. Lanes 1 and 2 are samples from the NICD AIDS Virus Research Unit. Lane 3 is the Negative Control (Neg). The best amplification was seen with the 10µl input.

2.3.2 Denaturing of RNA before starting the reaction.

The RNA was denatured by incubation at 65°C and 70°C. Figure 2.2 show the results obtained after electrophoresis on a 0.8% agarose gel. A sample from the NICD AIDS Virus Research Unit was amplified with negative HIV-1 plasma used as a Neg. A reaction with water used as template substitute was used for a negative template control (NTC). The best amplification was seen with the 65°C and 70°C denaturing step than with the experimental setup without a prior denaturing step. This suggests higher yields of PCR product with denaturing of RNA than without.

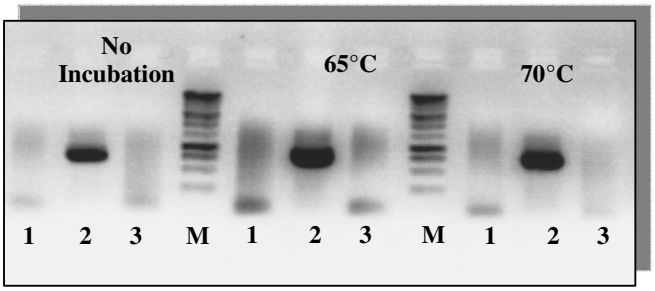

Figure 2.2: Denaturing of RNA before performing PCR reaction. Lane 1-Negative control (NEG), Lane 2-Sample, and Lane 3- No template control (NTC). The same samples were used for all 3 the PCR amplifications. Better amplification was seen, when RNA was denatured at 65°C and 70°C than without denaturing.

2.3.3 Titration of magnesium and primer for the first and second round PCR concentrations

A primer and magnesium checkerboard titration was performed to determine the optimal concentrations for amplification. For the optimisation reaction sample DR10 from the NICD AIDS Virus Research Unit was amplified. HIV-1 plasma was used as a Neg. Figure 2.3 shows the second round titration result after electrophoresis on a 0.8% agarose gel. The PCR products of the optimisation by checkerboard titration could only be visualized on an ethidium bromide containing 0.8% agarose gel after the second round PCR due to no visible bands on the agarose gel from the first round PCR reaction. The conditions as described in section 2.2.2.4 were used for the first round PCR.

A 40pmol concentration for primers and a 2mM concentration for magnesium were selected as the best amplification could be seen at these concentrations for the second round PCR of the RT fragment. The PR fragment had the best amplification results at 40pmol concentration for primers and a 2.5mM concentration for magnesium with good amplification seen at 2mM.

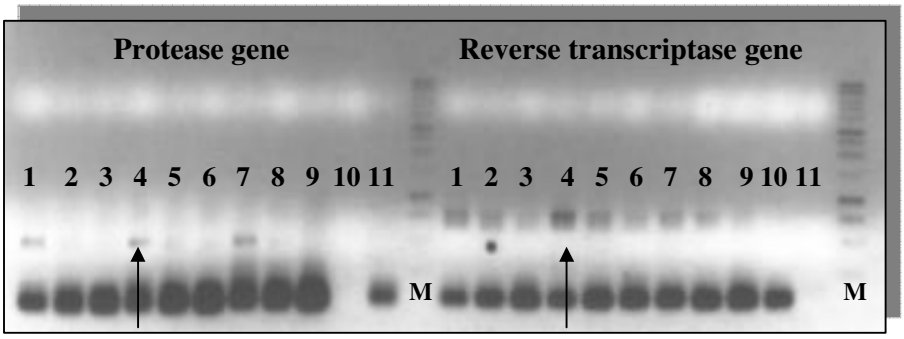

Figure 2.3: Titration of Mg^+ and primer concentration for first round PCR.
Lane 1 = primer 40pmoles and Mg^+ 1.5mM, Lane 2 = primer 80pmoles and Mg^+ 1.5mM, Lane 3 = primer 120pmoles and Mg^+ 1.5mM, Lane 4 = primer 40pmoles and Mg^+ 2mM, Lane 5 = primer 80pmoles and Mg^+ 2mM, Lane 5 = primer 120pmoles and Mg^+ 2mM, Lane 7 = primer 40pmoles and Mg^+ 2.5mM, Lane 8 = primer 80pmoles and Mg^+ 2.5mM, Lane 9 = primer 120pmoles and Mg^+ 2.5mM, Lane 10 = Negative control and Lane 11 = Non template control.

2.3.4 Validation

2.3.4.1 Sensitivity and specificity testing

2.3.4.1.1 Analytical sensitivity

To ensure that the assay could detect low copy numbers, the 6 samples from the NICD AIDS Virus Research Unit were used in the sensitivity testing. The number of isolates detected for the RT gene at each copy number input was 6/6 at 1000 copies/ml, 5/6 at 100 copies per ml and 3/6 at 10 copies per ml. Using a probit regression model the limit of detection for the RT gene PCR was estimated to be 112 (37-881; 95%CI) copies/ml. The number of isolates detected for the PR gene at each copy number input was 6/6 at 1000 copies/ml, 4/6 at 100 copies per ml and 3/6 at 10 copies per ml. Using a probit regression model the limit of detection for the in-house assay was estimated to be 190 (67-1524; 95%CI) copies/ml.

2.3.4.1.2 Specificity of the assay

All resultant sequences corresponded 100% to the HIV-1 *pol* gene according to the NCBI Blast algorithm which indicated the high specificity of the primers used.

2.3.4.2 Comparison of samples from the NICD AIDS Virus Research Unit

All six the isolates from the NICD AIDS Virus Research Unit could be amplified and sequenced using the TFIH assay.

There was a 100% concordance between RT gene mutations that we detected and the sequences of the three samples, which were published by Pillay *et al* (2008). These samples were all HIV-1 subtype C. Only the RT gene was sequenced by the NICD AIDS Virus Research Unit and no results were made available for the PR gene. No resistance mutations were detected with the TFIH assay for the PR gene. No NRTI resistance mutations were detected in samples NVP8b, NVP64 and NVP186. The results for NVP8b were the same as supplied by the NICD AIDS Virus Research Unit. The results from the other 2 samples did not agree. The mutations found in sample NVP64 differed from the expected results and had the same mutations and subtype than sample NVP8b. The results also differed for NVP186 with no mutations detected by the TFIH assay. These samples were retested to exclude human errors. The same results were obtained with the repeat test. The results obtained for samples NVP8b, NVP64 and NVP186 are summarised in Table 2.4.

Table 2.4: Resistance mutation detected in samples NVP8b, NVP64 and NVP186 from the NICD AIDS Virus Research Unit that did not correlate with TFIH

Sample	***Expected Resistance mutations for NNRTI**	**Resistance mutations obtained for NNRTI with the TFIH assay:**	**[α]Subtype**
NVP8b	K103N	K103N	A1C
NVP64	V106A G190A	K103N	A1C
NVP186	K103N V179I G190A	None	A(A1)

*The expected results were supplied by Lynn Morris from the NICD AIDS Research Unit
[α]The Rega HIV-1 subtyping tool Version 2.0 (http://dbpartners.stanford.edu/Rega_Subtyping/) with jpHMM (jumping profile Hidden Markov Model) http://jphmm. gobics.de/ was used for determining the HIV-1 subtype

2.3.4.3 Comparison of Round Robin HIV-1 genotyping assessment system from the University of Würzburg.

All the samples could be amplified and sequenced by both the TFIH assay and the ViroSeq™ HIV-1 genotyping system. In Table 2.5 a summary of the results obtained for both assays is shown. The expected results for the Round Robin HIV-1 genotyping assessment system are listed in Appendix C. Although all the samples received from the Round Robin HIV-1 genotyping assessment system could be amplified with both TFIH assay and the ViroSeq™ HIV-1 genotyping system, the quality of the sequences obtained for WQC1 was bad and the T69i insertion was missed by both assays. The V108IV mutation in WQC5 was missed by the TFIH assay and the E44DE mutation in WQC6 was missed by the ViroSeq™ HIV-1 genotyping system. Although a few mutations were missed, both assays performed the same with the Round Robin HIV-1 genotyping assessment system and the performance of the TFIH assay was acceptable.

Table 2.5: Summary of results obtained from the Round Robin HIV-1 genotyping assessment system from the University of Würzburg. Samples are numbered WQC1 to WQC6.

Assays	**WQC1**	**WQC2**	**WQC3**	**WQC4**	**WQC5**	**WQC6**
ViroSeq	Missed 1 mutation Missed insertion **T69i**	100%	100%	Missed 1 mutation **N88D**	100%	Missed 1 mutation **E44DE**
Two fragment In-House	Missed 2 mutations Missed insertion **T69i** and polymorphism S68N	100%	100%	Missed 1 mutation **N88D**	Missed 1 mutation **V108IV**	100%

2.3.4.4 Testing with the Quality Control of Molecular Diagnostic system

All samples from the QCMD system could be amplified and sequenced with a score of 329 out of 332. The mutations detected are shown in Table 2.6.

Table 2.6: Resistance mutation detected by the TFIH assay in 5 samples received from the 2007 QCMD program

Sample	PI Major Mutations:	PI Minor Mutations:	NRTI Mutations:	NNRTI Mutations:	Insertions	*Subtype
QCMD 1	D30N N88D	L10F	M41L E44D D67N T69D M184V L210W T215Y	A98G		C
QCMD 2	None	None	D67N T69i L210W T215Y	K101E V106M G190A	RT AA Insertion: codon 69 AA: ES NA: GAGTCT	C
QCMD 3	M46I I54V V82A L90M	L10IV A71T	None	None		B
QCMD 4	M46I I54V V82A L90M	L10IV A71T	None	None		B
QCMD 5	None	None	None	None		F

*The Rega HIV-1 subtyping tool Version 2.0 (http://dbpartners.stanford.edu/Rega_Subtyping/) with jpHMM (jumping profile Hidden Markov Model) http://jphmm. gobics.de/ was used for determining the HIV-1 subtype

Chapter 3

Chapter 3

3. Detection of resistance mutations in mothers from the Western Cape, who received a regimen of single dose nevirapine (sd NVP) combined with a short course zidovudine (AZT) as part of prevention of mother to child transmission (PMTCT).

3.1 Introduction

The prevelance of NVP resistance after an sd-NVP in PMTCT programs was found to be in 19%-69% with a significant difference in different HIV-1 subtypes as the highest percentages are seen in subtype D (36%) and C (46-69%) (Eshleman *et al*, 2005 and Kurle *et al*, 2007). The aim of this study in part was to detect if dual therapy, a short course of AZT with sd-NVP, will be associated with a lower prevalence of resistance mutations in maternal patients included in the Western Cape PMTCT program.

3.2 Materials and methods

In this section a short overview of the materials and methods used not described in Chapter 2, Section 2.2 will be described.

3.2.1 Sample cohort

Approval for the project was obtained from the Ethics Committee of the University of Stellenbosch (Committee of Human Research) on 17 January 2005. The project number is N04/09/146. The letter of approval is attached in Appendix E. Samples were collected from pregnant women who took part in the Western Cape PMTCT program and visited the Tygerberg Obstetrics Clinic and Delft Community Hospital. These woman all received AZT during pregnancy from 34 weeks and NVP at delivery. Sample collection started from February 2005 and continued until 116 samples have been obtained. Informed consent for drawing 10 ml of EDTA blood was obtained to measure CD4-cell count, viral load, and to do genotyping for viral subtype and the presence of resistance mutations. Information on prior exposure to antiretroviral therapy was also collected. At the follow-up visit EDTA blood was drawn from the mother to determine the presence of resistance mutations and 2ml of EDTA blood was drawn from the baby for a HIV-1 PCR.

3.2.2 Sample preparation

Patient whole blood samples were used in this study for determining viral load, CD4 cell count and to do genotyping. Approximately 3ml plasma from the mother and 1ml plasma from the baby was stored, in 1 ml aliquots, from the EDTA blood received for this study. Plasma was separating from the red blood cells with centrifugation at 3000 revolutions per minute (rpm) for 10 minutes. The samples were stored at –70°C until analysis. RNA extraction was performed as described in 2.2.2.2.2.

3.2.3 Determination the CD4 cell count

The Pan Leucogating (Beckman Coulter Inc., USA) was used to determine the CD4 cell count assay, as part of routine testing in the Tygerberg NHLS Immunology laboratory.

3.2.4 Statistical Analysis

The Mann-Whitney U Test was used for statistical analysis to compare CD4 counts and $\log_{10}$ HIV-1 viral loads in patients with or without resistance.

3.3. Results

The results of this study were published in Van Zyl *et al* (2008) Appendix F.

3.3.1 Samples

The maternal patients characteristics are set out in Appendix G. Patients were included for analysis on the basis that a baseline on the first visit to the clinic and a post partum follow-up specimen were collected. Follow-up visits were scheduled for six weeks post partum but due to logistical difficulties this could not always be achieved and let to variation in the follow-up intervals. The median follow-up time was 7 weeks (3 to 19 weeks). Follow-up samples were collected from 107 mothers. Using the TFIH assay, both samples from 97 of the 107 mothers could be amplified with PCR and complete sequences for the reverse transcriptase gene could be obtained for them. Due to the variation in follow-up times only 76 mothers were included in the study. The mothers that were included had follow-up samples collected within 60 days postpartum and administration of NVP. From the 76 mothers, 62 (82%) had samples collected between 4 and 8 weeks postpartum.

3.3.2 Viral load (VL) and CD4 cell count

A summary of the results can be seen in Table 3.1. VL results were available at baseline for 74 of the 76 patients. The VL were performed on the Nuclisens EasQ® assay (bioMérieux, Boxtel, Netherlands), which has a 95% hit rate at the lower limit of detection of 357 copies/ml for 1 ml input (Yao *et al*, 2005). The CD4 were performed on The Pan Leucogating (Beckman Coulter Inc., USA) assay. CD4 cell counts were available for all 76 patients include in the study.

Table 3.1: VL and CD4 cell count results obtained for 76 samples included in the study

Sample Code	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Sample Code	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)
RP 1.1	02/28/05	*LDL	353	**RP 105.1**	05/03/06	5600	365
RP 1.2	08/04/05			**RP 105.2**	07/18/06		
RP 7.1	03/02/05	14000	286	**RP 106.1**	05/03/06	1500	252
RP 7.2	06/30/05			**RP 106.2**	07/18/06		
RP 8.1	03/04/05	7900	365	**RP 107.1**	05/03/06	11000	289
RP 8.2	08/31/05			**RP 107.2**	07/20/06		
RP 9.1	03/07/05	1200	624	**RP 111.1**	05/15/06	34000	181
RP 9.2	06/30/05			**RP 111.2**	07/18/06		
RP 10.2	03/10/05	5700	717	**RP 113.1**	05/15/06	510	697
RP 10.2	06/08/05			**RP 113.2**	08/25/06		
RP 12.1	03/14/05	3200	625	**RP 116.1**	05/22/06	5100	603
RP 12.2	11/04/05			**RP 116.2**	08/15/06		
RP 13.1	03/14/05	5800	329	**RP 121.1**	05/29/06	17000	226
RP 13.2	07/08/06			**RP 121.2**	08/01/06		
RP 17.1	03/22/05	5200	344	**RP 122.1**	06/05/06	14000	261
RP 17.2	07/28/05			**RP 122.2**	08/22/06		
RP 19.1	03/24/05	12000	404	**RP 124.1**	06/05/06	46000	655
RP 19.2	08/18/05			**RP 124.2**	08/22/06		
RP 25.1	04/04/05	6800	357	**RP 126.1**	06/07/06	5400	518
RP 25.2	08/31/05			**RP 126.2**	08/29/06		
RP 28.1	04/04/05	9300	405	**RP 128.1**	06/12/06	29000	316
RP 28.2	06/08/05			**RP 128.2**	09/12/06		
RP 29.1	04/06/05	17000	201	**RP 129.1**	06/12/06	25000	300
RP 29.2	07/08/05			**RP 129.2**	08/29/06		
RP 35.1	04/23/05	20000	733	**RP 131.1**	06/19/06	510	658
RP 35.2	08/01/05			**RP 131.2**	08/22/06		
RP 37.1	05/10/05	2000	417	**RP 133.1**	06/14/06	1400	738
RP 37.2	10/05/05			**RP 133.2**	09/14/06		
RP 40.1	06/08/05	59000	317	**RP 134.1**	06/19/06	14000	188
RP 40.2	10/05/05			**RP 134.2**	08/25/06		
RP 41.1	06/28/05		365	**RP 135.1**	06/19/06	6300	209
RP 41.2	12/05/05			**RP 135.2**	08/29/06		

Table 3.1 continue: VL and CD4 cell count results obtained for 76 samples included in this study

Sample Code	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Sample Code	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)
RP 46.1	09/07/05	210000	255	**RP 138.1**	06/26/06	13000	384
RP 46.2	12/05/05			**RP 138.2A**	06/26/07		
RP 47.1	09/08/05	29000	297	**RP 138.2B**	10/31/06		
RP 47.2	12/05/05			**RP 139.1**	06/26/06	4000	335
RP 48.1	09/08/05	3200	651	**RP 139.2**	08/29/06		
RP 48.2	02/20/06			**RP 140.1**	06/28/06	1400	625
RP 49.1	09/08/05	8600	737	**RP 140.2**	08/24/06		
RP 49.2	12/05/05			**RP 143.1**	06/28/06	29000	248
RP 70.1	03/08/06	290000	168	**RP 143.2**	09/28/06		
RP 70.2	04/20/06			**RP 144.1**	07/10/06	2600	597
RP 71.1	03/13/06	LDL	502	**RP 144.2**	09/14/06		
RP 71.2	06/06/06			**RP 145.1**	07/10/06	63000	342
RP 72.1	03/13/06	630	595	**RP 145.2**	09/14/06		
RP 72.2	07/06/06			**RP 149.1**	07/19/06	12000	613
RP 73.1	03/13/06	330000		**RP 149.2**	09/28/06		
RP 73.2	05/30/06			**RP 150.1**	07/09/06	1400	564
RP 74.1	03/15/06	LDL	239	**RP 150.2**	10/26/06		
RP 74.2	05/12/06			**RP 151.1**	07/19/06	1700	428
RP 75.1	03/22/06	9000	341	**RP 151.2**	09/28/06		
RP 75.2	06/01/06			**RP 152.1**	07/19/06	14000	206
RP 76.1	03/22/06	LDL	789	**RP 152.2**	09/28/06		
RP 76.2	05/30/06			**RP 156.1**	07/26/06	1000	587
RP 77.1	03/22/06	740	233	**RP 156.2**	09/27/06		
RP 77.2	06/09/06			**RP 157.1**	07/26/06	2600	498
RP 79.1	03/22/06	940	654	**RP 157.2**	10/05/06		
RP 79.2	06/08/06			**RP 159.1**	08/16/06	9700	380
RP 83.1	03/29/06	1700	443	**RP 159.2**	10/26/06		
RP 83.2	07/04/06			**RP 162.1**	08/30/06	2000	204
RP 86.1	04/03/06	8600	345	**RP 162.2**	11/07/06		
RP 86.2	06/15/06			**RP 165.1**	09/11/06	65000	878
RP 87.1	04/03/06	59000	587	**RP 165.2**	11/21/06		
RP 87.2	06/07/06			**RP 166.1**	09/13/06	3600	261
RP 88.1	04/03/06	1100	390	**RP 166.2**	11/23/06		
RP 88.2	07/06/06			**RP 167.1**	09/13/06	3300	334
RP 91.1	04/05/06	54000	510	**RP 167.2**	11/09/06		
RP 91.2	06/15/06			**RP 169.1**	09/13/06	6900	526
RP 96.1	04/19/06	710	1018	**RP 169.2**	01/19/07		
RP 96.2	07/11/06			**RP 172.1**	18/09/06		295
RP 99.1	04/19/06	29000	243	**RP 172.2**	17/11/06		
RP 99.2	07/04/06			**RP 173.1**	06/20/06	12000	372
RP 100.1	04/19/06	36000	553	**RP 173.2**	11/09/06		
RP 100.2	07/06/06			**RP 178.1**	10/18/06	3400	364
RP 101.1	04/24/06	57000		**RP 178.2**	01/10/07		
RP 101.2	07/04/06						

*LDL: Viral load that was lower than detection limit

A summary of the mean CD4 count and VL analysis is shown in Table 3.2. The mean CD4 count, given in cells per microliter (cells/µl), in the NVP resistant group was 353 versus 458 in the NVP sensitive group; a difference which is not statistically significant (p=0.07, Mann-Whitney U Test). Geometric means of VL for the resistant and sensitive groups respectively were 4 652 (range LDL to 290 000) and 5 250 (range LDL to 330 000) copies/ml. This difference was not statistically significant (p= 0.94; Mann-Whitney U Test for log VL).

Table 3.2: A summary of the CD4 count and VL results of mothers testing sensitive and resistant to NVP on follow-up

Parameter	Resistant (n=13)	Sensitive (n=63)	p value
Mean CD4 count (range)	353 (168-651) cells per microliter	458 (181-1018) cells per microliter	0.07
Geometric mean of Viral load (range)	4 652 (LDL -290 000)	5 250 (LDL -330 000)	0.94

3.3.3 Genotyping assay

Since commercial genotypic testing is expensive, the TFIH assay optimized from Jacobs *et al* (2008) was used for genotyping. Readable sequences could be obtained for 97 of 107 (90.7%) patients with the TFIH assay. Figure 3.1 show a representation of the PCR product for the RT fragment on a 0.8% agerose gel after electrophoresis.

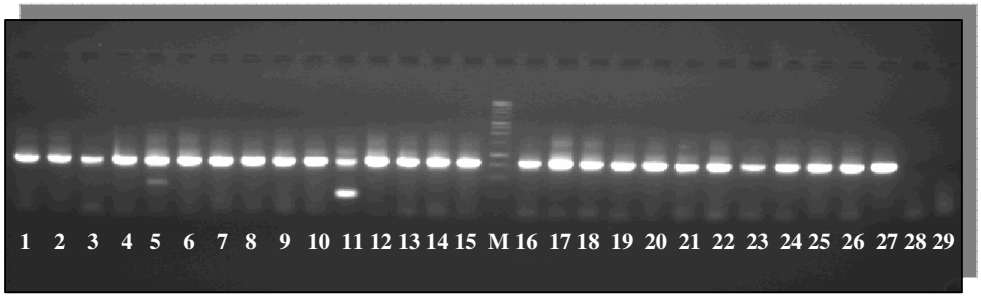

Figure 3.1: A representation of the PCR for sequencing of the RT fragment. The fragments obtained were purified using the QIAquick Spin Kit (QIAGEN GmbH, Germany) and sequenced using the ABI Prism® BigDye™ Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Warrington, UK). Lanes: Lane M = 1kb DNA Marker, Lanes 1-26 = Patient samples, Lane 27=PC, Lane 28-29 = Neg and NTC. In 2 of the samples two amplification bands are visible. The RT band is 804 bp of length while other unknown amplified fragments and are present at ±500 bp and ±250 bp.

3.3.4 Sequencing analysis for resistance mutations

Of the 76 sequences 75 were HIV-1 subtype C and 1 was HIV-1 subtype B by using the Rega Subtyping Tool Version 2.0 from the Stanford HIVdb website (http://dbpartners.stanford.edu/RegaSubtyping/). The mutations detected per patient are shown in Table 3.3. None of the baseline specimens showed NRTI resistance. One patient showed the K70R mutation at follow-up, which confers low-level AZT resistance.

Table 3.3: Resistance mutations detected

Patient	Baseline mutations (n=1)	Mutations on follow-up (n=16)	*Resistance	Follow-up interval (Days after delivery)	Viral load Baseline sample tested	CD4 Baseline sample tested
1	none	G190E	High-level	22	3200	625
2	none	V108I	Potential low-level	55	5200	344
3	none	K103N	High-level	49	17000	201
4	none	K103N; Y181C	High-level	44	210000	255
5	none	K103N	High-level	30	3200	651
6	none	K103N	High-level	20	290000	168
7	none	K101E	Low-level	52	[α]LDL	502
8	none	V108I	Potential low-level	35	LDL	239
9	none	K103N	High-level	34	1100	390
10	none	K103N; V106A	High-level	28	29000	243
11	none	K103N	High-level	44	14000	261
12	A98G	A98G; Y181C	High-level	22	4000	335
13	none	K70R; K101E	Low-level	32	9700	204

*According to the Stanford HIV Resistance Database Algorithm
[α]LDL = Below the lower detection limit

One of the 76 patients tested at baseline, the A98G mutation, confers low level resistance to NVP. The patient acquired the mutation Y181C after NVP exposure. In total, 13 out of the 76 (17.1%; 95% confidence interval (CI) 8.64- 25.5) patients had resistance mutations against NVP on the follow-up specimen, including the patient with a mutation at baseline. A total of 17 mutations were detected. The frequencies of mutations detected in the 76 patients are shown in Figure 3.2. The mutation most commonly detected was K103N in 7 patients with other NNRTI-associated mutations that included K101E, Y181C, V106A, V108I, and G190E. High-level NVP resistance were detected in 9 of the 13 patients with resistance mutations. The mutations present were K103N, Y181C, G190E or V106A, alone or in combination with other mutations. Low-level resistance was detected in 2 of the 13 patients with the K101E mutation present and potential low-level resistance was detected in 2 of the 13 patients with the V108I mutation present. The Stanford HIV Resistance database Algorithm was used to interpret resistance (Update 2007). One of the patients that developed resistance mutations received NVP previously while 4 patients that previously received NVP did not develop any resistance mutations. The Genbank accession numbers for 149 sequences produced by the TFIH assay are EU283624-EU283773.

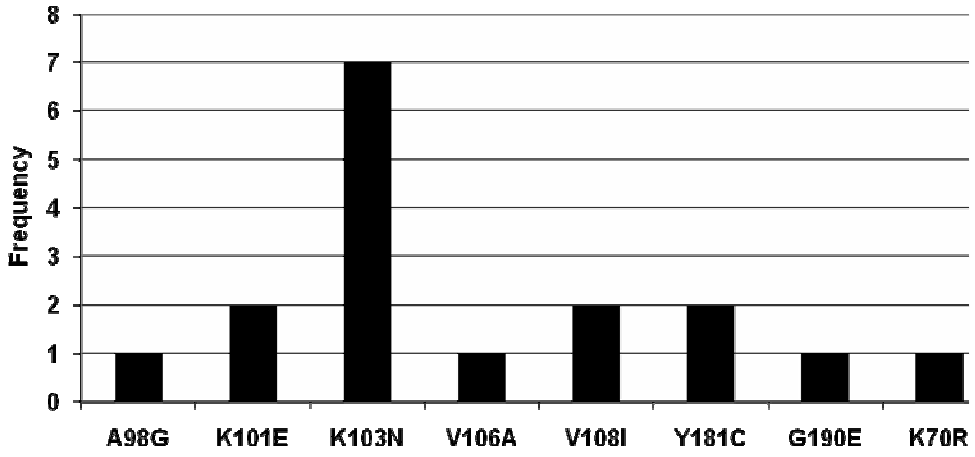

Figure 3.2: Resistance mutations detected in follow-up specimens

Chapter 4

Chapter 4

4. The further optimisation, validation and quality assurance of the in-house HIV-1 antiretroviral resistance tests to be more applicable and practical for routine resistance testing in a diagnostic setting

4.1 Introduction

Genotypic antiretroviral resistance testing, which is based on PCR and automated sequencing, has been used widely as a tool to monitor antiretroviral treatment of HIV-1 positive patients in industrialised countries. (EuroGuidelines for HIV resistance, 2001, Hirsch *et al*, 2000). Public funded therapy programmes in resource limited countries cannot afford resistance testing using commercial genotyping assays such as the ViroSeq™ Genotyping system V2.0. (Celera Diagnostics, CA, USA) and the Trugene™ HIV-1 genotyping System (Visible Genetics, Inc., Toronto, Canada). Nevertheless, there is a need for affordable and reliable genotyping testing in the developing world as resistance testing is valuable for patient management in choosing the best initial regimen or regimen after antiretroviral failure and for antiretroviral resistance surveillance, which can inform therapy programs.

The aim of this part of the study was to further adapt, validate and implement the TFIH assay for use in a diagnostic setting: The National Health Laboratory Services (NHLS). The following aspects of the TFIH assay (Appendix D) were investigated to obtain a less technical and more cost effective assay: extraction method, primer selection for first and second round PCR, addition of dithiothreitol (DTT), increased annealing temperature and decreased reagent volume in second round PCR and improved primer selection for sequencing. A validation of the Improved In-House (IIH) assay was completed using QC samples comparing them with the TFIH and the ViroSeq™ Genotyping system V2.0. The assays were accredited by South African National Accreditation System (SANAS) and implemented as a diagnostic assay in 2008.

4.2 Material and Methods

In this section a short overview of the materials and methods not detailed in Chapter 2, Section 2.2 and Chapter 3, Section 3.2, are described. The TFIH assay optimised and validated in Chapter 2 was initially used for subsequenet optimitaion. Unless

stated the same protocol was follow for the IIH assay. A comparison of the methods used in the TFIH assay and the IIH assay are listed in Table 4.1.

Table 4.1: A comparison of the methods used in the TFIH assay and the IIH assay.

Steps in genotyping assay	**TFIH**	**IIH**
Plasma used for extraction	1ml	1ml
RNA Extraction	QIAamp UltraSens Kit	QIAamp UltraSens Kit
First round PCR		
Denature of RNA	RNA denatured	RNA denatured
Input volume	10µl	10µl
*Additives	None	DTT
*Number of primers used	4	2
Second round PCR		
Input volume	2µl	2µl
*Reaction volume	50µl	25µl
*Number of primers used	4	2
*Annealing temperature	55ºC	60ºC
Sequencing reactions		
*Volume of BigDye™ Terminator v3.1 ready reaction mix	4µl	1µl
Sequencing primers	8	8
*Annealing temperatures	45ºC and 50ºC	50ºC

* Differences between assays

4.2.1 Sample

4.2.1.1 ViroSeq™ HIV-1 genotyping assay

Twenty nine samples received for routine diagnostic resistance testing at the NHLS, Department of Virology, Tygerberg Hospital were used for comparison of the IIH assay and the ViroSeq™ HIV-1 genotyping assay.

4.2.1.2 Two Fragment In-House assay

Sixty seven samples received for routine diagnostic resistance testing at the NHLS, Department of Virology, Tygerberg Hospital were used for comparison of the IIH assay and the TFIH assay.

4.2.3 RNA extraction

The QIAamp® UltraSens virus kit (QIAGEN GmbH, Germany), the QIAamp® viral RNA mini kit (QIAGEN GmbH, Germany) and the NucliSense EasyMag automated extraction systems (bioMérieux, Boxtel, Netherlands) were investigated for RNA extraction using different input and elution volumes. With the QIAamp UltraSens Kit (QIAGEN GmbH, Germany) RNA was extracted from 1ml sample and eluted in 60µl. With the QIAamp® viral RNA mini kit (QIAGEN GmbH, Germany) RNA was extracted from 140µl of sample and a final elution volume of 60µl was obtained. With the NucliSense EasyMag automated extraction systems RNA was extracted from 1ml sample and eluted in 60µl. For viral load detection RNA was extracted from a 1ml sample and eluted in 25µl. Sample DR10 from the NICD AIDS Virus Research Unit was used. Ten-fold dilutions of the sample were made in plasma to a 1 in 1000 dilution. One ml of each of the dilutions was used to extract RNA on each of the extraction systems evaluated. The viral loads of the dilutions were determined. A TFIH PCR was performed on RNA from each of the extraction systems as well as the RNA that was extracted for viral load quantification. The PCR product was visualized with gel electrophoresis on a 0.8% agarose gel.

4.2.4 Dithiothreitol (DTT)

DTT is a reducing agent that reduces disulfides quantitatively (Ausubel *et al*, 2006). It was found that DTT affects enzyme activity with reduction and is used in RNA amplification methods (Jeon *et al*, and Snoeck *et al*, 2006). Dithiothreitol (DDT) was added as an enhancer.

4.2.5 *gag* PCR

A nested PCR was performed to obtain a PCR product from the *gag* gene to determine if the samples for comparison studies were amplifiable. The Access RT-PCR System (Promega, Madison, USA) was used for the first round PCR reactions in a one step PCR protocol. The PCR primers used (Swanson *et al*, 2003) are listed in Table 4.2. Five microliter of RNA was added to the first round reaction mix consisting of AMV/T*fl* 5X reaction buffer at a 1X concentration, dNTPs mix at 0.2mM each, primers at 40pmol, 2.5mM $MgSO_2$, AMV RT 0.1U/µl, T*fl* DNA Polymerase 0.1U/µl, and nuclease-free water up to a final volume of 50µl. The reaction was preformed using a 9700 PCR system ((Applied Biosystems, Foster City, USA) using the 9600 ramp speed. The reaction mixtures were prepared in 0.2ml PCR tube (QSP, Porex BioProducts Inc, California, USA). The following cycles were used for first round PCRs. Incubation for one cycle at 48°C for 45 minutes to enable reverse transcription and then a cycle at 94°C for 2 minutes to inactivate AMV Reverse transcriptase and allowing denaturing of the DNA. For second strand cDNA and amplification the reaction was cycled 40 times at 94°C for 30 seconds to denature, at to 53°C for 30 seconds to anneal the primers and at 72°C for 60 seconds to extend the product. A last extension for at 72°C for 10 minutes was done. The reaction was then stored at 4°C until the second round reactions could be done. The *Taq* Go-Taq® Flexi DNA polymerase (Promega, Madison, USA) was used for the second round PCR reaction mixture using the first round PCR product. The PCR primers (Swanson *et al*, 2003) are listed in Table 4.2. A volume of 3µl of the first round PCR reaction was added two the second round reaction mix that consisted of 5X buffer at a 1X concentration, PCR Nucleotide mix (Promega, Madison, USA) at 0.2mM each, primers at 40pmol each, 1.5mM $MgCl_2$, Taq DNA Polymerase 0.5U/µl, and nuclease-free water up to a final volume of 50µl. The following cycles were used for second round PCR reaction mixtures: 40 cycles of 94°C for 30 seconds to denature DNA, 56°C for 30 seconds for primer annealing and 68°C for 30 seconds for primer extension. This is followed by a final 7 minutes at 68°C to complete primer extension. The product was then stored at 4°C until the second round reactions could be done.

Table 4.2: Primers for *gag* PCR

First round primers	**HBX2**	**Primer Tm**	**Primer Direction**	**Primer Sequence**
P24-1	1177-1193	47.1°C	*Forward*	5' - AGY CAA AAT TAY CCY ATA GT - 3'
P24-7	1826-1841	56.3°C	*Reverse*	5' – CCC TGR CAT GCT GTC ATC A – 3'
Second round primers				
M/O p24-2	1240-1256	54.2°C	*Forward*	5' – AGR ACY TTR AAY GCA TGG GT – 3'
M/O p24-6	1703-1718	55.2°C	*Reverse*	5' - TGT GWA GCT TGY TCR GCT C – 3'

4.2.4 ViroSeq™ HIV-1 genotyping assay

The ViroSeq™ HIV-1 Genotyping system V2.0. (Celera Diagnostics, CA, USA) was used as a reference assay and performed according to the manufacturer's instructions (including the extraction method).

4.2.5 Assembly and analysis of sequencing results

Sequencing alignment was done using the Sequencer software V4.7 (Gene Codes Corporation, Ann Arbor USA). The Stanford HIVdb program for Genotyping resistance interpretation was used to detect drug resistance mutations. http://hivdb.stanford.edu/ index.html, Updated January 2007.

4.3 Optimization of the Improved In-House assay

RNA extraction methods were evaluated, different primers for both the PCR and the sequencing reactions were selected, DTT was used as an additive to enhance the RT-PCR, the reaction volume of the second round PCR was decreased, and the BigDye™ Terminator v3.1 ready reaction cycle sequencing reactions were adapted for better cost effectiveness.

4.3.1 Primer selection

4.3.1.1 Primer selection for PCR to obtain complete fragment

A schematic representation shows the primer selection in Figure 4.1. The TFIH assay used four primers to amplify the PR and the RT gene separately to obtain two PCR products that have to be sequence with separate primer sets. For practical reasons a primer set were selected to amplify one fragment for sequencing. Four of the same primers used in the TFIH assay were selected for the IIH assay. The first and second

round forward primers of the PR PCR fragment with the reverse primers of the RT PCR fragment from the TFIH assay was selected to obtain one fragment of the *pol* gene for sequencing. The PCR primers are listed in Table 4.3. The samples from the NICD AIDS Virus Research Unit were used to perform a first and second round PCR according to protocol in Appendix D using the primers selected.

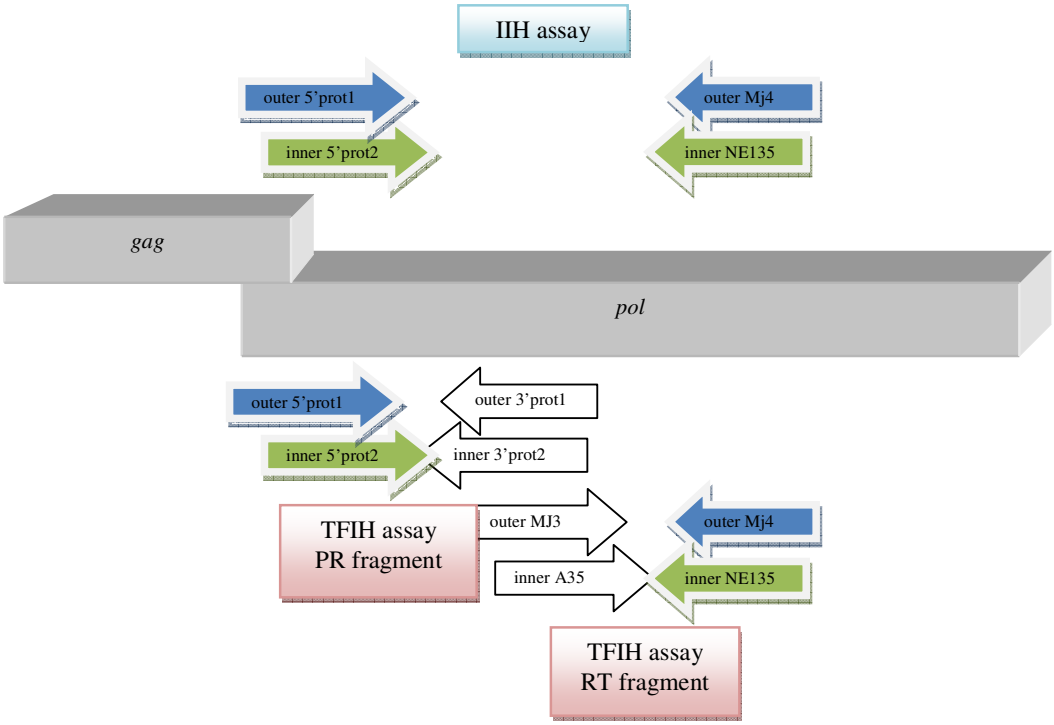

Figure 4.1: Schematic representation of the Primer selection for IIH assay. The figure is not drawn to scale.

Table 4.3: Primers used for IIH assays genotyping PCR

First round Primers	HBX2 number	Primer Tm	Primer direction	Primer Sequence
outer 5'prot1	2082-2109	57.0°C	*Forward*	5'- TAA TTT TTT AGG GAA GAT CTG GCC TTC C -3'
outer Mj4	3399-3420	54.8°C	*Reverse*	5'- CTG TTA GTG CTT TGG TTC CTC T –3'
Second Round Primers				
inner 5'prot2	2136-2163	68.2°C	*Forward*	5'- TCA GAG CAG ACC AGA GCC AAC AGC CCC A –3'
inner NE135	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3

4.3.1.2 Selection of primers for sequencing to anneal at same temperature

The primers used for sequencing of the TFIH assay had two different annealing temperatures, 50°C and 45°C. This complicated the practical performance of the sequencing, since sequencing reactions could not be performed on one thermocycler at the same time. The reactions had to be prepared at separate times or two separate thermocyclers were required to accommodate the difference in primer annealing temperature. Different primers, as seen in Table 4.4, were selected and tested to find those that annealed at the same temperature with the same effectiveness.

Table 4.4: Primer tested for use in sequencing reaction in the IIH assay.

Primer	HBX2 number	Primer Tm	Primer Direction	Primer Sequence	References
JA204	2138-2159	67.9°c	*Forward*	5' – TTC AGA GCA GAC CAG AGC CAA CAG C – 3'	Lindström and Albert, 2003
inner 5'prot2	2139-2163	68.2°C	*Forward*	5'- TCA GAG CAG ACC AGA GCC AAC AGC CCC A –3'	Plantier *et al*, 2005
pol1D	2254-2271	56.3°C	*Forward*	5'- TCC CTC AAA TCA CTC TTT GGC -3'	Loxton, 2004
outer Mj3	2483-2499	54.3°C	*Forward*	5- AGT AGG ACC TAC ACC TGT CA –3'	Plantier *et al*, 2005
ABB20F2	2470-2486	49.7°C	*Forward*	5' – TAG GTA CAG TGT TAG TAG GA – 3'	Personal Communication, John Hackett
F2054	2511-2528	53.4°C	*Forward*	5' – GGR CGA AAT ATG TTG ACT CAG – 3'	Personal Communication, John Hackett
inner A35	2533-2564	58.4°C	*Forward*	5'- TTG GTT GCA CTT TAA TTC CCA TTA GTC CTA TT –3'	Plantier *et al*, 2005
AK11	2574-2592	48.4°C	*Forward*	5' - GTA CCA GTA AAA TTA AAR CCA G - 3'	Lindström and Albert, 2003
pol rev 2D	2588-2599	49.0°C	*Reverse*	5' – CCA TCC ATT CCT GGC – 3'	Loxton, 2004
JA217	2622-2643	49.5°C	*Reverse*	5'- CTT TTA TTT TTT CTT CTG TCA ATG G -3'	Lindström and Albert, 2003
outer 3'prot1	2703-2731	57.6°C	*Reverse*	5'- GCA AAT ACT GGA GTA TTG TAT GGA TTT TCA GG –3'	Plantier *et al*, 2005
pol 3D rev	2869-2881	48.5°C	*Reverse*	5'- CCC ACA TCC AGT ACT G – 3'	Loxton, 2004
pol 3	2885-2901	63.4°C	*Forward*	5' - GGG GGA TGC ATA TTT TTC AG –3'	Personel communication, Susan Engelbrecht
AK12	2947-2944	50.6°C	*Reverse*	5' - TGG TGT YTC ATT RTT TRY ACT AG – 3'	Lindström and Albert, 2003
ABB20-3F	2983-2999	51.6°C	*Forward*	5' – ATC AGT ACA ATG TGC TTC CA - 3'	Personal Communication, John Hackett
inner NE135	3300-3331	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3	Plantier *et al*, 2005
JA205	3311-3335	61.7°C	*Reverse*	5' – TTT TCC CAC TAA CTT CTG TAT GTC ARR G – 3'	Lindström and Albert, 2003
AK10	3316-3333	50.5°C	*Reverse*	5' - TYC CCA CTA AYT TCT GTA TRT C –3'	Lindström and Albert, 2003

4.3.2 Addition of Dithiothreitol (DTT) to obtain a more sensitive PCR

To investigate if the addition of DTT enhances the performance of the PCR, reaction mixes was prepared as described in Appendix D with the primers used described in Table 4.3. DTT was added to the first round reaction mix at a concentration of 0.8mM as described in the reference assay.

Sample DR10 received from the NICD AIDS Virus Research Unit was used to prepare six 10 fold dilutions to determine if the addition of DTT would influence the amplification of RNA. These dilutions were amplified in parallel reactions mixtures with and without DTT.

4.3.3 Changes made to the second round PCR

Production of *Taq* DNA Polymerase in storage buffer B system (Promega, Madison, USA), which was used to optimise The TFIH assay, was discontinued by the manufacture and replaced with Go-*Taq*® Flexi DNA polymerase (Promega, Madison, USA). This improved polymerase system was implemented in the IIH assay.

4.3.3.1 Increased annealing temperature of the second round PCR

The melting temperatures of the primers for the IIH assay in the second round PCR are listed in Table 4.3. In the TFIH assay, the annealing temperature for the second round PCR was 55° due to the lower melting temperatures of 2 primers (see Table D.1 in Appendix D). The possibility of increasing the melting temperature of the second round PCR to 60°C was investigated by performing the PCR with an annealing temperature of 60°C in parallel with 55°C using 10 fold dilutions of sample DR10 from the NICD AIDS Virus Research Unit.

4.3.3.2 Decreased reaction mix volume and an increased cDNA input volume in the second round PCR

A combination of DTT and Go-*Taq*® Flexi DNA polymerase improved the PCR reaction. Subsequently a good quality PCR product was obtained in a 50µl reaction. To save on costs and to increase sensitivity of the second round PCR reaction, the possibility of a smaller reaction mix volume was investigated. The PCR reaction mix described in the TFIH assay was reduced to a final volume of 25µl with a 2 µl input of first round PCR product. This was compared to the PCR reaction mix of TFIH

assay at 50µl with a 2µl input using 10 fold dilutions of sample DR10 from the NICD AIDS Virus Research Unit.

4.3.4 Sequencing buffer to reduce cost of sequencing reaction

The sequencing buffer supplied by Applied Biosystems, Warrington, UK was used to reduce the cost of sequencing by reducing the amount of ABI Prism® BigDye™ Terminator v3.1 ready reaction mix needed in a reaction. Sequencing reactions were performed using the terminator ready reaction mix diluted with sequencing buffer in a 1 to 3 dilution.

4.4 Validation

Validation of the IIH assay was performed according to the protocol described in Appendix H using six HIV-1 samples obtained from the NICD AIDS Virus Research Unit, the six samples obtained from the Round Robin HIV-1 genotyping assessment system as well as the samples obtained from the Quality Control of Molecular Diagnostic system.

4.4.1 Sensitivity and specificity testing

4.4.1.1 Analytical sensitivity

Six samples obtained from the NICD AIDS Virus Research Unit were used to determine the analytical sensitivity. The viral loads of the isolates were determined with the NucliSense Easy Q HIV-1 system V1.2 (Biomérieux bv, Boxtel). Ten fold dilutions form 100 000 to 1 copies/ml was prepared. Viral RNA was extracted with the QIAamp® UltraSens™ Virus Kit (QIAGEN GmbH, Germany) and amplified with IIH assay to obtain a 1200 bp region of the *pol* gene coding for the *protease* (PR) and approximately 260 amino acids of the *reverse transcriptase* (RT) enzymes. In order to determine the limit of detection we used a probit regression model in R (version 1.11.1).

4.4.1.2 Specificity

To verify the specificity of this IIH assay, the *pol* gene sequences obtained, were submitted to BLAST (as described in section 2.2.3) of the National Center of Biotechnology information (http://www.ncbi.nlm.nih.gov).

4.4.1.3 Comparison with the NICD AIDS Virus Research Unit Samples

The six HIV-1 isolates obtained from the NICD AIDS Research Unit were amplified and sequenced with the IIH assay.

4.4.1.4 Comparison with the Round Robin HIV-1 genotyping assessment system

The six QC samples obtained from the Round Robin HIV-1 genotyping assessment system were analysed in parallel with the IIH assay, the TFIH assay as described in Appendix D and the ViroSeq™ HIV-1 Genotyping system.

4.4.1.5 Quality Control of Molecular Diagnostic system (QCMD)

The TFIH assay as described in Appendix D was enrolled in the QCMD external quality assessment system for evaluation. The samples were also tested with the IIH assay as describe in Appendix H, for comparison with the TFIH assay. The IIH assay was enrolled in the QCMD external quality assessment system for evaluation in 2008.

4.4.1.5 Comparison of methods

EDTA whole blood samples received for routine drug resistance testing at the NHLS Tygerberg Hospital, Department of Virology, were separated from the red blood cells with centrifugation at 3000 revolutions per minute (rpm) for 10 minutes and plasma was stored in 1ml aliquots at -70°C.

4.4.1.5.1 *gag* PCR to determine if the samples are amplifiable

A nested PCR was performed to obtain a PCR product from the *gag* gene to determine if the samples for comparison studies were amplifiable.

4.4.1.5.2 ViroSeq™ HIV-1 genotyping assay

Twenty nine samples were tested in parallel on the IIH assay and the ViroSeq™ HIV-1 genotyping system.

4.4.1.5.3 Two Fragment In-House assay

Sixty seven samples were tested in parallel on the IIH assay and the TFIH assay described in Appendix D.

4.5 Results

4.5.1 Optimisation of IIH assay for use in routine diagnostic HIV-1 drug resistance testing

The following section describes the results obtained after investigating different aspects of the genotyping assay to improve cost effectiveness and technical efficiency.

4.5.1.1 Extraction of viral RNA for genotyping

A summary of the detection of RNA after extraction with the QIAamp® UltraSens virus kit (QIAGEN GmbH, Germany), the QIAamp® viral RNA mini kit (QIAGEN GmbH, Germany) and the NucliSense EasyMag automated extraction systems (bioMérieux, Boxtel, Netherlands) are shown in Table 4.5.

Table 4.5: Detection of RNA with different extraction methods

	Elution Volume	**10000 copies/ml**	**1000 copies/ml**	**100 copies/ml**
QIAamp® UltraSens virus kit	60 µl	+	+	+
QIAamp® viral RNA mini kit	60 µl	+	+	-
NucliSense EasyMag automated extraction systems	60 µl	+	+	-
NucliSense EasyMag automated extraction systems RNA from Viral load extraction	25 µl	+	+	+

Sample DR10 from the NICD AIDS Virus Research Unit was used for extraction and an HIV-1 negative plasma sample was included as a Neg. A non template control (NTC) was included. The QIAamp® UltraSens virus kit performed the best of all the assays with a final elution of 60µl. The NucliSense EasyMag automated extraction systems with a 60µl elution did not perform well, as it could not amplify the sample with 100 copies/ml. Weak amplification bands were visible with the naked eye in samples with higher copy numbers (Figure 4.2). The NucliSense EasyMag automated extraction systems with a 25µl elution performed as well as the QIAamp® UltraSens virus kit. A disadvantage of the method was the small volume of RNA available for further use. The QIAamp® viral RNA mini kit (QIAGEN GmbH, Germany) with a 60µl elution did not perform well as it could only detect 1000 copies/ml. The best

assayfor optimization will be the QIAamp® UltraSens virus kit as it has the best results with a larger elution volume of 60µl.

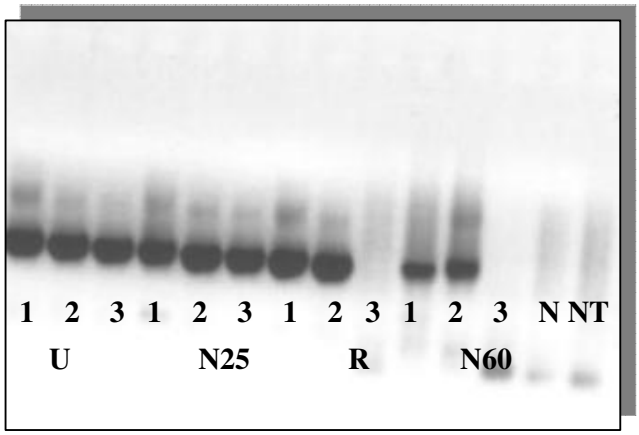

Figure 4.2: Agarose gel of PCR product obtained from RNA extracted with different extraction methods. U=QIAamp® UltraSens virus kit, N25=NucliSense EasyMag with 25µl elution, N60=NucliSense EasyMag with 60µl elution, R=QIAamp® viral RNA mini kit. Lane 1=10000 copies/ml, Lane 2=1000 copies/ml, Lane 3=100 copies/ml, N=Negative control, NT=Non template control.

4.5.1.2 Primer selection

4.5.1.2.1 Primer selection for PCR to obtain a complete fragment

For practical reasons, one set of primers that can amplify one PCR product for sequencing was selected. The first and second round forward primers of the PR PCR with the reverse primers of the RT PCR from the TFIH assay were selected to obtain one PCR fragment. Figure 4.3 shows a representation of the complete fragment amplification visualised on 0.8% agarose gel by electrophoresis. This fragment amplified the same region as the PR and RT assay and obtains a sequence of approximately 1200 base pairs for analysis of resistance mutations in the PR and RT genes. An HIV-1 negative plasma sample was included as a Neg along with a NTC.

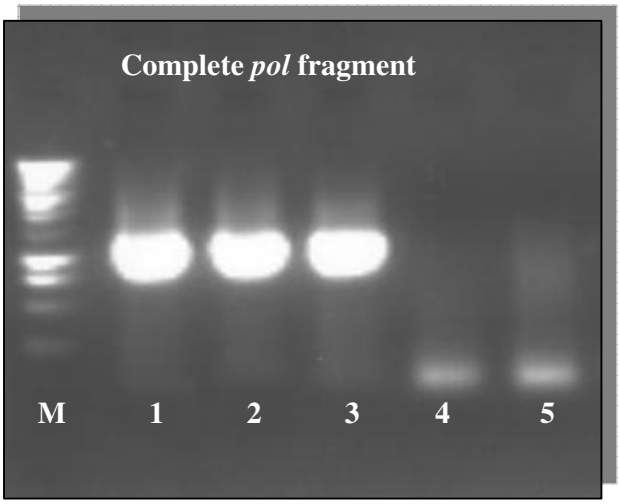

Figure 4.3: A complete fragment of 1200 base pairs was obtained by using the first and second round forward primers of the PR PCR with the reverse primers of the RT PCR from the TFIH assay. Lane 1-3 = Known HIV-1 positive patient samples, N = Negative Control and NTC = Non Template Control.

4.5.1.2.2 Selection of primers to anneal at the same temperature

Primers were selected for sequencing of PCR product to obtain a set of sequencing primers that can be annealed at 50°C to produce a high quality sequence for genotyping analysis. The primers are in Table 4.6. With the TFIH assay the two PCR products were sequenced with three and five primers respectively (see Figure 4.1). One of the sequencing primers for the PR assay had an annealing temperature of 45°C and the other two annealed at 50°C. One of the sequencing primers of the RT assay anneal at 45°C and the other four annealed at 50°C. There were two annealing temperatures for sequencing and two separate PCR reactions had to be performed, necessitating two instruments or a longer time to completion on one instrument. This caused a delay in sequencing. Primers with an annealing temperature of 50°C were selected for sequencing. A list of the primers selected is shown in Table 4.6. By selecting primers at the same annealing temperature one sequence amplification cycle could be performed leading to more efficient time management and fewer errors.

Table 4.6: Primer selected for use in sequencing reaction of the IIH assay.

Primer	HBX2 number	Primer Tm	Primer Direction	Primer Sequence	References
pol1D	2254-2271	56.3°C	*Forward*	5'- TCC CTC AAA TCA CTC TTT GGC -3'	Loxton, 2004
F2054	2511-2528	53.4°C	*Forward*	5' – GGR CGA AAT ATG TTG ACT CAG – 3'	Personal Communication, John Hackett
AK11	2574-2592	48.4°C	*Forward*	5' - GTA CCA GTA AAA TTA AAR CCA G - 3'	Lindström and Albert, 2003
JA217	2622-2643	49.5°C	*Reverse*	5'- CTT TTA TTT TTT CTT CTG TCA ATG G -3'	Lindström and Albert, 2003
pol 3	2885-2901	63.4°C	*Forward*	5' - GGG GGA TGC ATA TTT TTC AG –3'	Personal Communication, Susan Engelbrecht
inner NE135	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3	Plantier *et al*, 2005
ABB20-3F	2983-2999	51.6°C	*Forward*	5' – ATC AGT ACA ATG TGC TTC CA - 3'	Personal Communication, John Hackett
AK10	3316-3333	50.5°C	*Reverse*	5' - TYC CCA CTA AYT TCT GTA TRT C –3'	Lindström and Albert, 2003

4.5.1.3 Addition of Dithiothreitol (DTT) to obtain a more sensitive PCR

DTT was added to reaction mixtures to enhance the sensitivity of the PCR reaction. Tenfold dilutions of RNA from NVP8b sample of the NICD AIDS Virus Research Unit were prepared and amplified with the protocol form Appendix G. With addition of DTT, RNA could be detected down to a concentration of 100 copies/ml (Figure 4.4). The PCR reaction without the DTT could only detect RNA down to 1000 copies/ml. An HIV-1 negative plasma sample was included as a Neg.

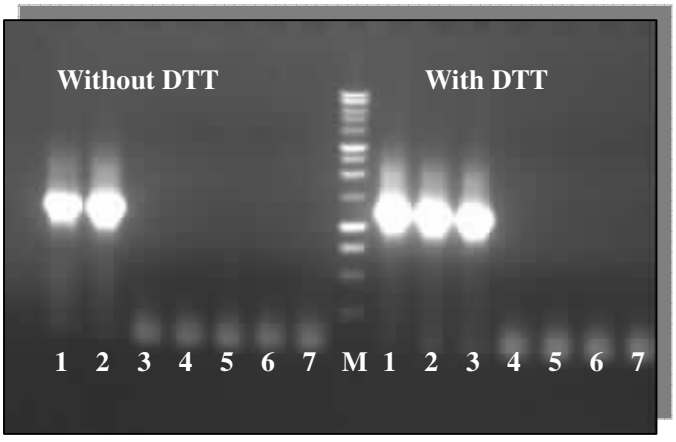

Figure 4.4: Tenfold dilutions of sample amplified with PCR reaction mixes without DTT and with DTT. M=Marker, 1=100 000 copies/ml, 2=1000 copies/ml, 3=100 copies/ml, 4=10 copies/ml, 5=1 copies/ml, 6=0 copies/ml, 7=Negatice Control.

4.5.1.4 Changes to second round PCR

4.5.1.4.1 Increased annealing temperature of the second round PCR

The primer annealing temperature for the second round PCR of the IIH assay was increased because the newly selected primers had a higher melting temperature than the primers used in the TFIH. A second round PCR was performed with an annealing temperature of 60°C to increase the specificity and sensitivity of the second round PCR reaction. RNA could be detected down to a concentration of 100 copies/ml with the annealing temperature of 60°C. With the annealing temperature of 55°C, RNA could be detected down to 1000 copies/ml with no amplification visible at 100 copies/ml. Figure 4.5 shows PCR products, for different inputs and annealing temperatures after gel electrophoresis on a 0.8% agarose gel. An HIV-1 negative plasma sample was included as a Neg along with a NTC.

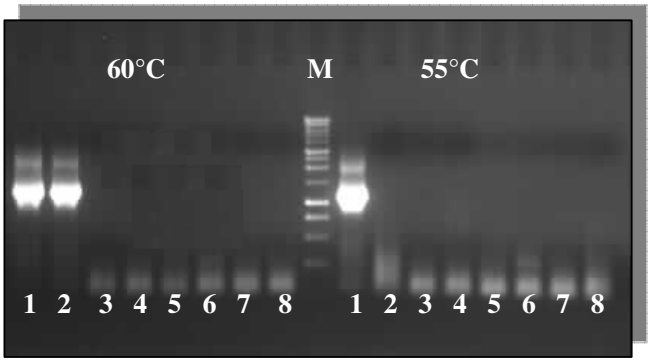

Figure 4.5: Agarose gel of PCR reaction with a 60°C and 55°C annealing temperature. M=Marker, 1=1000 copies/ml, 2=100 copies/ml, 3=10 copies/ml, 4=1 copies/ml, 5=0 copies/ml, 6=0 copies/ml, 7=Negative Control, 8=Non Template Control. RNA could be detected up to a 1000 copies/ml with a 60°C annealing temperature and only 1000 copies/ml with a 55°C annealing temperature.

4.5.1.4.2 Decrease of reaction mix volume with increased cDNA input in second round PCR

To decrease the cost of the PCR and to increase the sensitivity of the assay the reaction volume of the second round PCR was decreased to 25µl with an input of 2 µl of first round PCR product. Keeping the input volume at 2µl increased the concentration of cDNA input and thus possibly the sensitivity of the assay. RNA could be detected up to a 100 copies/ml dilution of the sample with both PCR reaction mixes and the amplification bands expressed on 0.8% agarose gels are more prominent with the lower reaction volume and higher input as shown in Figure 4.6.

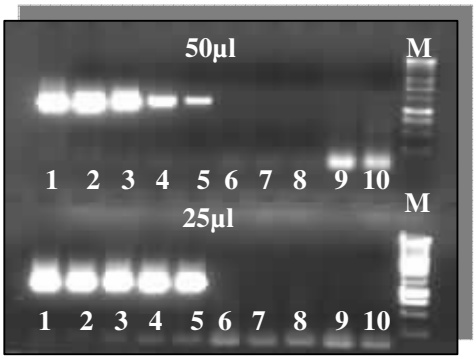

Figure 4.6: Tenfold dilutions of sample amplified in 50µl and 25µl PCR reaction with a 2µl input visualized on a agarose gel. M=Marker, 1=1 000 000 copies/ml, 2=100 000 copies/ml, 3=10 000 copies/ml, 4=1000 copies/ml, 5=100 copies/ml, 6=10 copies/ml, 7=1 copies/ml, 8=0 copies/ml, 9=0 copies/ml 10=Negative Control.

4.5.1.5 Sequencing buffer to reduce cost of sequencing reaction

To reduce the cost of the sequencing reactions a 3 fold dilution of the ABI Prism® BigDye™ Terminator v3.1 ready reaction mix with sequencing buffer, supplied free of cost by Applied Biosystems, Warrington, UK, were used to obtain sequences. These were analysed with the Sequencer software V4.7. By using sequencing buffer the amount of sequencing reaction that could be performed with the in-house assay increased 3 fold and this lead to lower assay cost.

4.5.2 Sensitivity and specificity testing

4.5.2.1 Analytical sensitivity

The number of isolates detected at each copy number input was 6/6 at 1000 copies/ml, 5/6 at 100 copies per ml, 2/6 at 10 copies per ml and 1/6 at 1 copy per ml. Using a probit regression model the limit of detection for the in-house assay was estimated to be 137 (87-794; 95%CI) copies/ml.

4.5.2.2 Specificity of the assay

All PCR products as sequenced corresponded to the HIV-1 *pol* gene.

4.5.3 Validation

4.5.3.1 Testing with samples from the NICD AIDS Virus Research Unit

All the samples from the NICD AIDS virus Research Unit could be amplified and sequenced. The results obtained correlated a 100% with that of the TFIH assay described in section 2.3.4.4.

4.5.3.2 Round Robin HIV-1 genotyping assessment system from the University of Würzburg

All the samples could be amplified and complete sequences were obtained with the IIH assay. The assay could detect all except one the resistance associated mutations, insertion T69i, and polymorphism S68N as seen in Table 4.7. The performance of the assay was better than the VirSeq™ HIV-1 genotyping system and the TFIH assay.

Table 4.7: Summary results for the Round Robin HIV-1 genotyping assessment system for the VirSeq™ HIV-1 genotyping system, the TFIH assay and the IIH assay

	WQC1	**WQC2**	**WQC3**	**WQC4**	**WQC5**	**WQC6**
***Subtype**	B	B	G	B	B	B
ViroSeq™	1 mutation missed	100% Correlation	100% Correlation	1 mutation missed	100% Correlation	1 mutation missed
TFIH	1 mutation missed	100% Correlation	100% Correlation	1 mutation missed	1 mutation missed	100% Correlation
IIH	1 mutation missed	100%100% Correlation	100% Correlation	100% Correlation	100% Correlation	100% Correlation
Comment	WQC 1 sequence was of poor quality for all assays. Only fragments could be analysed. All missed insertion **T69i** and polymorphism S68N					

*The Rega HIV-1 subtyping tool Version 2.0 (http://dbpartners.stanford.edu/Rega_Subtyping/) with jpHMM (jumping profile Hidden Markov Model) http://jphmm. gobics.de/ was used for determining the HIV-1 subtype

4.5.3.3 Quality Control of Molecular Diagnostic system (QCMD)

All samples received from the 2007 QCMD program could be amplified and sequenced. The resistance mutations detected corresponds to the ones obtained with the TFIH assay as discussed in section 2.3.4.4 and shown in Table 2.7, Chapter 2. All but one of the samples could be amplified by the IIH for the 2008 QCMD program with a score of 266 out of 266. The resistance mutation detected by the IIH assay in 5 samples is listed in Table 4.8.

Table 4.8: Resistance mutation detected by the IIH assay in 5 samples received with the 2008 QCMD program

Sample	PI Major Mutations:	PI Minor Mutations:	NRTI Mutations:	NNRTI Mutations:	Insertions	*Subtype
QCMD 1	None	None	None	None		G
QCMD 2	D30N N88D	L10F T74S	M41L E44D D67N T69D M184V L210W T215Y	A98G		C
QCMD 3	M46I I54V V82A L90M	L10IV A71T	None	None		B
QCMD 5	None	None	D67N T69i L210W T215Y	K101E V106M G190A	RT AA Insertion: codon 69 AA: ES NA: GAGTCT	C

*The Rega HIV-1 subtyping tool Version 2.0 (http://dbpartners.stanford.edu/Rega_Subtyping/) with jpHMM (jumping profile Hidden Markov Model) http://jphmm. gobics.de/ was used for determining the HIV-1 subtype

4.5.3.4 Comparison between assays

4.5.3.4.1 *gag* PCR to determine if comparison samples are amplifiable

4.5.3.4.1.1 *gag* PCR amplification of samples for comparison between IIH assay with the ViroSeq™ HIV-1 genotyping assay

The 29 samples selected for the comparison between the IIH assay and the ViroSeq™ HIV-1 genotyping assay were tested with the *gag* PCR to determine if they are amplifiable. All the samples could be amplified with the *gag* PCR. The results are shown in Table 4.9.

Table 4.9: The results of the *gag* PCR amplification. All samples could be amplified

Sample	*gag* PCR result	Sample	*gag* PCR result
330314	Pos	333676	Pos
330315	Pos	333679	Pos
330316	Pos	333983	Pos
330318	Pos	333985	Pos
330818	Pos	334791	Pos
330819	Pos	335286	Pos
330820	Pos	335287	Pos
330822	Pos	335291	Pos
330823	Pos	335294	Pos
331101	Pos	335299	Pos
331502	Pos	335301	Pos
332071	Pos	336514	Pos
332885	Pos	336517	Pos
333599	Pos	336844	Pos
333601	Pos		

*Pos=Positive for amplification
Neg=Negative for amplification

4.5.3.4.1.2 *gag* PCR amplification of samples for comparison between IIH assay with the TFIH assay

The 67 samples, selected for the comparison between the IIH assay and the TFIH assay, were tested with the *gag* PCR to determine if they are amplifiable. Five of the samples could not be amplified with the *gag* PCR. The results are set out in Table 4.10.

Table 4.10: The results of the *gag* PCR amplification. Five samples could not be amplified, highlighted in grey

Sample no.	*gag* PCR result	Sampleno.	*gag* PCR result	Sample no.	*gag* PCR result
144745	*Pos	149518	Pos	Aretas 22	Pos
144771	Pos	150142	Pos	Aretas 23	Pos
144831	Pos	150143	Neg	Aretas 25	Pos
145426	□Neg	151267	Pos	Aretas 24	Pos
145427	Pos	151450	Pos	Aretas 29	Pos
145881	Neg	151679	Pos	Aretas 30	Pos
145882	Pos	151680	Pos	Aretas 32	Pos
146142	Neg	151924	Pos	Aretas 43	Pos
146143	Neg	151925	Pos	Aretas 84	Pos
146593	Pos	152122	Pos	Aretas 88	Pos
147028	Pos	152529	Pos	Aretas 89	Pos
147642	Pos	152869	Pos	Aretas 90	Pos
147643	Pos	153545	Pos	Aretas 91	Pos
148090	Pos	153547	Pos	Aretas 92	Pos
148091	Pos	153549	Pos	Aretas 93	Pos
148092	Pos	153550	Pos	Aretas 94	Pos
148093	Pos	153551	Pos	Aretas 95	Pos
148221	Pos	153792	Pos	Aretas 96	Pos
148222	Pos	153793	Pos	Aretas 100	Pos
148824	Pos	Aretas 12	Pos	Aretas 101	Pos
148825	Pos	Aretas 19	Pos	Aretas 102	Pos
149004	Pos	Aretas 20	Pos		
149517	Pos	Aretas 21	Pos		

*Pos=Positive for amplification
□Neg=Negative for amplification

4.5.3.4.2 Comparison between IIH assay and the ViroSeq™ HIV-1 genotyping assay

The Improved in-house assay was compared with the ViroSeq™ HIV-1 genotyping assay by testing 29 samples in parallel. The patient's characteristics are in Appendix J. The ViroSeq™ HIV-1 Genotyping assay could amplify 25 out of 29 samples with 3 incomplete sequences obtained from the 25 samples amplified. One sample could not be sequence with the ViroSeq™ HIV-1 Genotyping assay. The IIH assay could amplify and sequence all 29 samples with one incomplete sequence obtained. All of the samples were found to be HIV-1 subtype C except for one sample that was HIV-1 Subtype D.

Primer failures for the IIH assay were as follows: JA217 (n=5), AK10 (n=2), HIV-AK11 (n=3), outer 3'prot1 (n=2) and Pol3 (n=1). Obtaining a consensus sequence with the IIH assay by assembly of sequences from both strands was not possible for

10% of the samples due to primer failure with only 1 incomplete sequence obtained. Primer failures for the ViroSeq™ HIV-1 Genotyping assay were as follows: Primer A (n=9), Primer B (n=2), Primer C (n=1), Primer D (n=25), Primer F (n=3), Primer G (n=4), and Primer H (n=9). With the ViroSeq™ HIV-1 Genotyping assay consensus sequence from both strands was not possible for 48% of the samples due to primer failure with 3 incomplete sequences obtained. Primers for the IIH assay failed in 31% of the samples compared to an 80% (excluding primer D) failure rate in the ViroSeq™ HIV-1 Genotyping assay.

4.5.3.4.2.1 Antiretroviral drug resistance associated mutations detected by the IIH assay with the TFIH assay

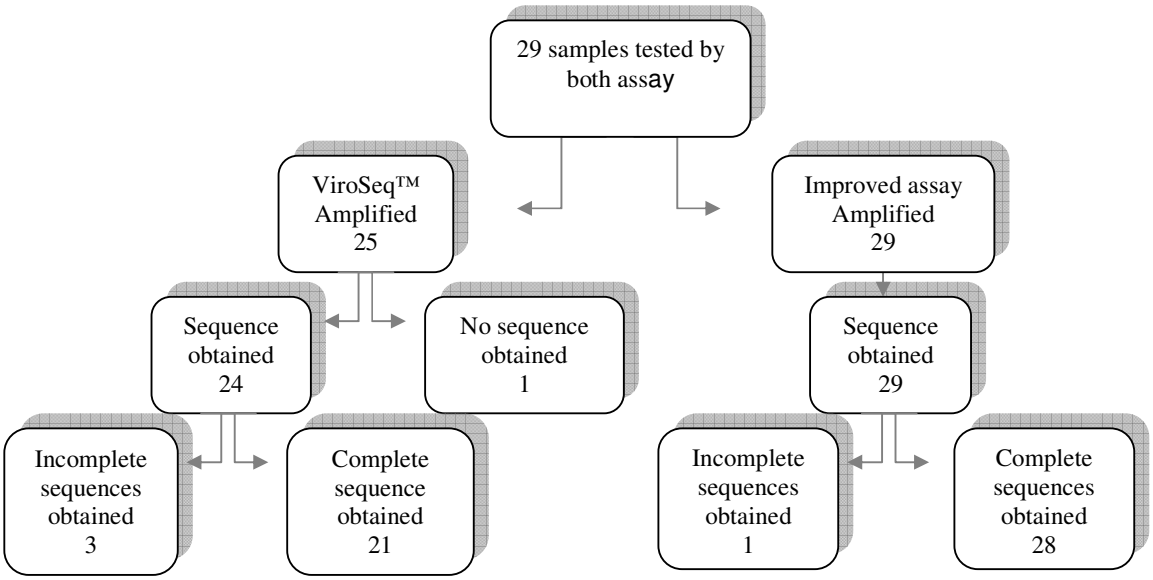

Figure 4.7: Schematic comparison of samples sequences with ViroSeq™ HIV-1 Genotyping assay and the IIH assays

A schematic comparison of the sequencing results are shown in Figure 4.7. Twenty five samples were sequenced with both the ViroSeq™ HIV-1 Genotyping assay and the IIH assays and a total of 94 and 105 mutation listed in the Stanford database were identified respectively by the ViroSeq™ HIV-1 Genotyping assay and the IIH assays. Identical resistance associated mutations were detected in 15 samples. A summary of the resistance mutations detected are shown in Figure 4.8.

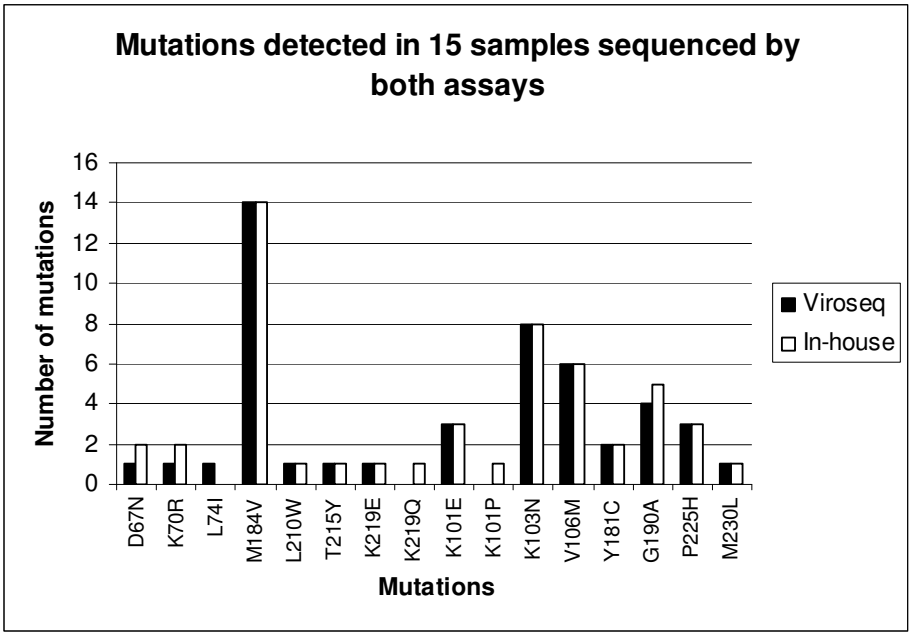

Figure 4.8: Comparison of mutations in 15 samples sequenced on both the In-house and the ViroSeq™ HIV-1 Genotyping assays.

Of the 6 samples with discrepant results, the ViroSeq™ HIV-1 Genotyping assay system detected 2 mutations not detected by the IIH assay. A summary of the mutations detected in each assay is given in Appendix I. The IIH assay detected 9 mutations that the ViroSeq™ HIV-1 Genotyping assay did not detect. A summary of the discrepant results are listed in Appendix K. The ViroSeq™ HIV-1 Genotyping assay and the IIH assay could each detect one mutation, in one case, that the other could not detect. Of the 11 discordant results, 8 were associated with drug resistance. No sequence could be obtained with the ViroSeq™ HIV-1 Genotyping assay for 1 sample, however a sequence could be obtained by the IIH assay and 10 mutations were detected of which 7 were associated with drug resistance (Table 4.11).

Table 4.11: Mutations in one sample sequenced with In-house assay only

PI	NRTI
L24I	L74V
L33F	Y115F
L10F	M184V
A71V	

The IIH assay could detect 15 mutations in the 4 samples that could not be amplified by the Viroseq, all these 4 samples harboured drug resistance mutations (Table 4.12). Incomplete sequences were obtained for 3 samples with the Viroseq assay and 1 sample with the IIH assay (Table 4.13). Most of the samples that could be sequenced were HIV-1 subtype C except for two HIV-1 subtype B sample.

Table 4.12: Mutations detected in 4 samples amplified with IIH assay only

	PI	**NRTI**	**NNRTI**
Sample 1		M184V	
Sample 2	T74S	A62AV M184V	V106M E138A V179D F227FL
Sample 3		M184MV	K101E V106M V108I G190A
Sample 4		M184V	K103N

Table 4.13: Mutations detected in 4 samples with incomplete sequences

	ViroSeq		**In House**		
	NRTI	NNRTI	PI	NRTI	NNRTI
Viroseq **Protease gene not sequenced**	K70EK M184V	A98G K103N P225H	T74S	K70EK M184V	A98G K103N P225H
Viroseq **82 AA Short on the Reverse Transcriptase gene**	M184V	V106M F227L		M184V	V106M F227L
Viroseq **62 AA Short on the Reverse Transcriptase gene**	M184V	K101E V106M G190A		M184V	K101E V106M G190A
IIH assay **24 AA Short on Protease gene**	M184V	K101P K103N		M184V	K101P K103N E138EK

4.5.3.4.3 Comparison between IIH assay and the TFIH assay

The IIH assay was compared to the TFIH assay by testing 67 samples in parallel. The characteristics of these patients are in Appendix L. A summary of the amplification of samples compared with the TFIH and IIH assay are shown in Table 4.14.

Table 4.14: A summary of the amplification of samples compared with the TFIH assay and the IIH assay

	TFIH assay	**IIH assay**	**Total**
Amplified	63 (94%)	57 (85%)	67
Complete sequence obtained	39 (62%)	53 (93%)	67
Incomplete sequences	24 (38%)	4 (7%)	-
Only PR sequence obtained	4 (1.5%)	NA	-
Only RT sequence obtained	8 (12%)	NA	-

The TFIH assay could amplify 63(94%) of 67 samples with 39 (62%) complete sequences and 24 (38%) incomplete sequences obtained from the amplified samples. The IIH assay could amplify 57 samples and complete sequences were obtained for 53 (93%) of samples with incomplete sequence obtained in only 4 (7%) samples. All the samples that could be sequenced were HIV-1 subtype C except for two HIV-1 Subtype B sample. A summary of the amplification and sequences obtained is shown in Table 4.15.

Table 4.15: A summary of amplification and sequence comparison between the TFIH assay and the IIH assay

Sample	***gag* PCR result**	**TFIH assay**	**Sequence obtained**	**IIH assay**	**Sequence obtained**
144745	Positive	[α]A	Complete	A	Complete
144771	Positive	A	Complete	A	Complete
144831	Positive	A	Fragments	A	Complete
145426	Negative	A	PR gene	No A	
145427	Positive	A	Complete	A	Fragments
145881	Negative	A	PR gene	No A	
145882	Positive	A	Fragments	A	Complete
146142	Negative	A	PR gene	No A	
146143	Negative	No A		No A	
146593	Positive	A	Complete	A	Complete
147028	Positive	A	Complete	A	Fragments
147642	Positive	A	Complete	A	Complete

Table 4.15 continue: A summary of amplification and sequence comparison between the TFIH assay and the IIH assay

Sample	*gag* PCR result	TFIH assay	Sequence obtained	IIH assay	Sequence obtained
147643	Positive	A	Complete	A	Complete
148090	Positive	A	Complete	A	Complete
148091	Positive	A	Complete	No A	
148092	Positive	A	Complete	A	Complete
148093	Positive	A	RT gene	A	Complete
148221	Positive	A	RT gene	A	Complete
148222	Positive	A	Complete	*No A	No Sequence
148824	Positive	A	Complete	□A	Fragments
148825	Positive	A	Complete	A	Complete
149004	Positive	A	Fragments	A	Complete
149517	Positive	A	Complete	A	Complete
149518	Positive	A	Complete	A	Complete
150142	Positive	A	Complete	A	Complete
150143	Negative	No A	No Sequence	No A	No Sequence
151267	Positive	A	RT gene	A	Complete
151450	Positive	A	Complete	A	Complete
151679	Positive	A	Complete	A	Complete
151680	Positive	A	Complete	A	Complete
151924	Positive	A	RT gene	A	Complete
151925	Positive	A	Fragments	A	Complete
152122	Positive	A	Complete	A	Complete
152529	Positive	A	RT gene	A	Complete
152869	Positive	A	RT gene	A	Complete
153545	Positive	A	Complete	A	Complete
153547	Positive	A	PR gene	A	Complete
153549	Positive	A	Fragments	A	Complete
153550	Positive	A	Complete	A	Complete
153551	Positive	A	Complete	No A	No Sequence
153792	Positive	A	Complete	A	Complete
153793	Positive	A	Complete	A	Complete
Aretas 12	Positive	A	Complete	A	Complete
Aretas 19	Positive	A	Fragments	A	Complete
Aeretas 20	Positive	A	Complete	A	Complete
Aeretas 21	Positive	A	Complete	A	Complete
Aeretas 22	Positive	A	Fragments	A	Complete
Aeretas 23	Positive	A	Complete	A	Complete
Aretas 25	Positive	A	Complete	A	Complete
Aeretas 24	Positive	A	Complete	A	Complete
Aeretas 29	Positive	A	Complete e	A	Fragments
Aeretas 30	Positive	A	Fragments	A	Complete
Aretas 32	Positive	No A	No Sequence	A	Complete
Aretas 43	Positive	A	RT gene	A	Complete
Aretas 84	Positive	A	Fragments	A	Fragments
Aretas 88	Positive	A	Complete	A	Complete
Aretas 89	Positive	A	Complete	A	Complete
Aretas 90	Positive	A	Fragments	A	Complete
Aretas 91	Positive	A	Complete	A	Complete

Table 4.15 continue: A summary of amplification and sequence comparison between the TFIH assay and the IIH assay

Sample	*gag* PCR result	TFIH assay	Sequence obtained	IIH assay	Sequence obtained
Aretas 92	Positive	A	Fragments	A	Complete
Aretas 93	Positive	A	Fragments	A	Complete
Aretas 94	Positive	A	Complete	A	Complete
Aretas 95	Positive	A	Complete	A	Complete
Aretas 96	Positive	No A	No Sequence	No A	No Sequence
Aretas 100	Positive	A	RT gene	A	Complete
Aretas 101	Positive	A	Complete	A	Complete
Aretas 102	Positive	A	Complete	A	Complete

*No A = No Amplification
[α]A = Amplification

The TFIH assay could amplify only the PR fragment in 4 samples. These samples could not be amplified with the *gag* PCR. The IIH assay could amplify 1 of these samples to obtain a PCR product for sequencing. The TFIH assay could amplify only the RT fragment in 8 samples and the IIH assay could amplify all of these samples to obtain product for sequencing. In one of these samples 2 major PI mutations were detected by the IIH assay.

Four samples could not be amplified by the TFIH assay, of which one sample could be amplified by the IIH assay. Two of these samples could not be amplified with the *gag* PCR. No amplification could be achieved in 10 samples with the IIH assay. Five of these samples could not be amplified by the *gag* PCR. In 4 of the 10 samples the TFIH assay could only amplify the PR fragment. In 4 samples that could not be amplified a complete fragment could be detected with the TFIH assay and 2 of the samples could not be detected by either assay. One sample could not be amplified by the TFIH assay but a complete fragment could be detected by the IIH assay.

4.5.3.4.3.1 Antiretroviral drug resistance associated mutations detected by the IIH assay with the TFIH assay

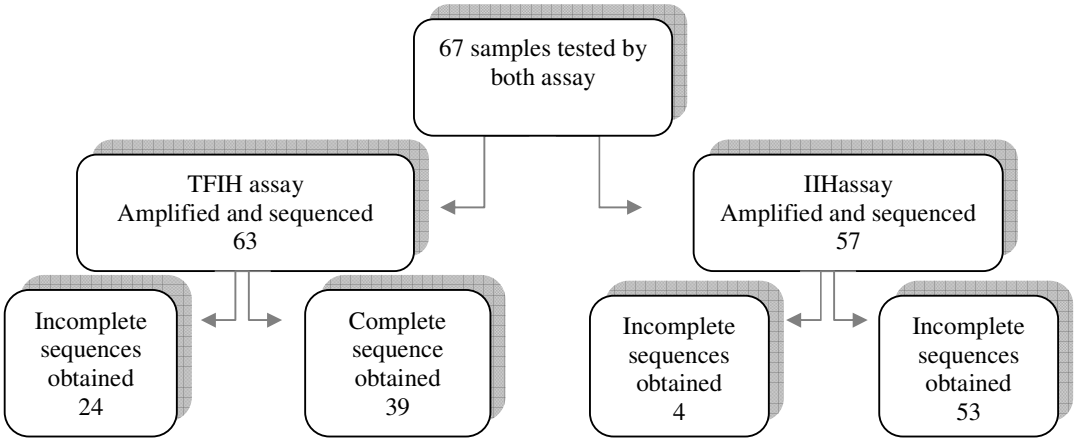

Figure 4.9: Schematic comparison of samples sequences with the IIH assay and the TFIH assays.

A schematic comparison of the sequencing results are shown in Figure 4.9. Complete sequences could be obtained with both the TFIH assay and the IIH assay in 38 samples and a total of 104 and 109 mutations listed in the Stanford database were identified respectively by the two assays. The resistance mutations detected are listed in Appendix M. Identical resistance associated mutations were detected in 31 samples. Eighteen samples had discrepant results. A summary of the samples with discordant resistance mutations are listed in Appendix N. Of the 18 samples with discrepant results the TFIH assay could detect 7 mutations not detected by the IIH assay. The IIH assay could detect 11 mutations that the TFIH assay did not detect. In 2 samples the TFIH assay and the IIH assay could both detect a mutation that the other could not detect. Of the 20 discordant mutations 14 were associated with drug resistance.

Incomplete sequences were obtained in 16 samples. Incomplete sequences were obtained with the TFIH assay in 12 samples, all of which could be sequenced completely with the IIH assay. In 4 samples incomplete sequences were obtained with the IIH assay that could be sequenced completely with the TFIH assay.

Primer failures for the TFIH assay were as follows R2051 (n=6), pol1D (n=7), JA217 7, pol3D (n=8), pol3rev 3, ABB20-3F (n=13), AK12 (n=7), AK11 (n=7) in 27 samples. Obtaining a consensus sequence with the assay from both strands was not

possible for 16 samples due to primer failure with 10 incomplete sequences obtained. Primer failures for the IIH assay were as follows: pol1D (n=0), AK11 (n=2), JA217 (n=2), outer 3'prot1 (n=2), pol 3 (n=5), AK10 (n=3), inner NE135 (n=4) and F2054 (n=15). Obtaining a consensus sequence with the assay from both strands was not possible for 4 samples due to primer failure with incomplete sequences obtained.

Chapter 5

Chapter 5

5.1 Discussion and Conclusion

5.1 Discussion

5.1.1 Introduction

This study has 3 objectives (1) to optimize a in-house assay for genotyping, published in Jacobs *et al* (2008), for a research and diagnostic tool, (2) to determine the resistance mutation from patient treated according to the provincial PMTCT protocol in the Western Cape, a sd-NVP, combined with a short course AZT and (3) to do further optimization and validation of the in-house assay to obtain a less technically difficult and more cost effect assay for the diagnostic setting.

5.1.2 The optimization and validation of a Two Fragment In-House HIV-1 antiretroviral resistance assay

The first aim of this study was to optimize and validate the TFIH assay for use in a diagnostic testing and research setting. The assay uses four primes to amplify the PR and the RT gene separately to obtain PCR products, of 487 and 804 base pairs respectively for sequencing. The two PCR products were sequenced with three and five primers respectively to sequence the complete PR and approximately 250 amino acids of the RT gene. The sequences generated are analysed and assembled with the Sequencer V4.7 software to obtain a consensus sequence of approximately 1200 base pairs for analysis of resistance mutations in two genes.

PCR is a tool with many different applications and no single PCR protocol is appropriate for all applications, therefore optimisation is always necessary when a new protocol is implemented. In the attempt to optimise the PCR, the following factors were investigated: the RNA and cDNA input volumes, the denaturing RNA before amplification and magnesium and primer concentrations. We investigated increasing the amount of input RNA as the amount of template influences the performance of the PCR. The input volume of the RNA template was increased from 5 µl to 10µl of RNA to increase the amplification rate in the first round PCR. To prevent inhibition of the second round PCR template, overload the input volume was reduced to 2µl. (Ausubel *et al*, 2006, Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010). There was better

amplification in the reactions with increased input, probably due to a higher yield of PCR product. The 10µl input in the first round probably contribute to the raised sensitivity of the TFIH assay with a high yield of PCR product for sequencing.

One of the problems encountered with RNA amplification is that during primer extension areas in the RNA are encountered that cause the reverse transcriptase to pause or terminate. The termination sites may be caused by secondary structures within the RNA. A high incubation temperature minimizes the effect of secondary structures (Ausubel *et al*, 2006). To eliminate termination sites that may be caused by secondary structures within the RNA, it should be denatured before amplification is performed. The ViroSeq™ HIV-1 Genotyping assay commercial system (Abbott Diagnostic Laboratories, Chicago, USA) suggest incubation at 65°C as the first step in the PCR and the Promega Impromp II (Promega, Madison, USA) assay also uses a incubation at 70°C as first step in RNA amplification. Both these methods were investigated for the TFIH assay. Better amplification was observed with denaturing of RNA than without. The two different methods of denaturing investigated at 65°C and 70°C. Both performed well. In this study the method of denaturing at 65°C was selected as the most simple to perform with no extra incubation on ice before amplification reactions could be performed.

A primer and magnesium titration was performed to optimise the concentrations for amplification. The concentration of the magnesium affects primer annealing, strand dissociation temperatures, product specificity, formation of primer-dimer artefacts and enzyme activity and also fidelity. The optimal magnesium concentration is dependent on the concentration of the dNTP's, primers and template. It is recommended that a PCR should contain 0.5-2.5 mM magnesium per reaction and that a titration must be done to determine the optimum concentration of magnesium for use (Innis *et al,* 1990).

An incorrect primer concentration may lead to mispriming and non-specific product formation. It also can increase the formation of primer-dimer artefacts. The formation of the aforementioned, compete with the selected target for enzyme, dNTPs and primers resulting in lower yields of product. A primer concentration between 0.1 and 0.5µM is the optimum for a PCR (Innis *et al,* 1990).

A problem encountered in this study was that the first round PCR, a one step reverse transcriptase PCR, did not deliver enough DNA for visualization on agarose gel. The titration was therefore performed on the second round PCRs. Both the RT and the PR PCR performed best at a 40pmol concentration of primer. The RT fragment had the best amplification results at 2mM concentration for magnesium will the PR fragment had the best amplification results at 2.5mM concentration with good amplification seen at 2mM. The original concentrations for the Jacobs *et al* (2008) assay used 2mM for both fragments. To keep the TFIH assay simple as possible the same conditions were used for both fragments.

The analytical sensitivity of the TFIH assay was determined to be 112 (37-881; 95%CI) copies/ml for the RT gene and 190 (67-1524; 95%CI) copies/ml for the PR gene. DNA blast of sequences showed that PCR products were specific for HIV-1. The sensitivity of the ViroSeq™ HIV-1 genotyping system is 1000 copies/ml (Manufacturers instructions).

Validation of the TFIH assay was done by testing samples received from the NICD AIDS Research Unit, comparing the TFIH assay with the ViroSeq™ HIV-1 genotyping system in the Round Robin HIV-1 genotyping assessment system and rolling the TFIH assay in the QCMD external quality assessment system. The results from the TFIH assay for the samples from the NICD AIDS Virus Research Unit published in Pillay *et al* (2008) correlated. The same resistance mutation could be detected. However the unpublished samples had discrepant results. NV8b and NV64 appear to be the same sample. Sample NV186 did not have any mutations present. The samples received and tested in this study were viral cultures while the sequence results supplied by the NICD AIDS Virus Research Unit were from plasma. There is a possibility that the cultured virus could have lost its mutations as it was under no antiretroviral drug pressure. Although all the samples received from the Round Robin HIV-1 genotyping assessment system could be amplified with both the TFIH assay and the ViroSeq™ HIV-1 genotyping system the quality of the sequences obtained for WQC1 was bad and the T69i insertion was missed by both assays. This sample was a recombinant virus and that could have contributed to the poor amplification (Bodem, 2007). The V108IV mutation in sample WQC5 was missed by the TFIH assay and the E44DE mutation in sample WQC6 was missed by the ViroSeq™ HIV-1 genotyping system. Although mutations were missed, both assays performed the same with the

Würzburg Quality Assessment system and the performance of the TFIH assay was considered acceptable.

The assay performed well with the QCMD system and scored 329 out of 332. All the mutations were detected with no mutations detected that were not included in the samples. The 3 points loss was due to base pair mixtures that were detected by the TFIH assay.

The TFIH assay showed good performance on the international assessment panels and the performance in the Round Robin HIV-1 genotyping assessment system was the same as the ViroSeq™ HIV genotyping assay. Satisfactory results were also obtained with the QCMD evaluations as all mutation present could be detected.

The cost off the commertial assay per sample is R4571.89. The TFIH assay can be completed for R 1636.88, with a total saving of R2935.01. The assay could be performed over a period of 4 days from extraction to the release of the final results. .

This assay can be used as a research and diagnostic resistance testing. An advantage of the TFIH assay is the use of the two fragments as separate assays for investigation of the PR and the RT gene, which could decrease the cost of a study.

5.1.3 Detection of resistance mutations in mothers from the Western Cape, who received a regimen of single dose nevirapine (sd NVP) combined with a short course zidovudine (AZT) as part of prevention of mother to child transmission (PMTCT).

The TFIH assay was used to determine the development of genotypic-antiretroviral resistance in patients treated according to the provincial PMTCT protocol in the Western Cape (sd-NVP, combined with a short course AZT). The resistance rate of 17.1% in a population that is predominant HIV-1 subtype C, was lower than seen in other studies done where sd-NVP alone was administered in HIV-1 subtype C positive patients in the PMTCT programs (Eshleman *et al*, 2005 and Kurle *et al*, 2007) which indicated that the dual therapy could reduce the risk of resistance to NVP. This might have been due to the fact the short course of AZT combined with NVP reduced the viral load to lower levels than sd-NVP alone with less viral replication and therefore a decreased likelihood of mutations (Chaix *et al*, 2007).

A potential bias towards under detection of resistance in this study is the relative lower baseline viral load and higher CD4 count in patients qualifing for PMTCT.

Patients with CD4 counts below 200 cells/µl qualified for HAART instead of this intervention. It was found that a higher viral load and low CD4 count are associated with the development of resistance (Eshleman *et al*, 2001 and Chaix *et al*, 2007).

Resistance mutations were found in 13 of the 76 samples included in this study and all patients with resistance mutations were infected with HIV-1 subtype C. Only one patient had a resistance mutation at baseline, this being the A98G mutation giving potential low level resistance to NVP. The A98G mutation is seen in 1% of drug naïve patients and in higher frequencies in HIV-1subtype C than in subtype B (Grossman *et al*, 2004). This patient acquired the Y181C mutation, causing high-level resistance to NVP, after receiving the PMTCT treatment. The K103N mutation that results in high-level resistance to NVP was found in 7 of the 13 patients. This has been reported as the most common mutation in sd NVP treatment in PMTCT by other studies as well (Flys *et al*, 2005, Eshelman (a) *et al*, 2004 and Eshelman (b) *et al*, 2004). The K103N mutations persist for a long period of time after sd-NVP (Flys *et al*, 2007, Palmer *et al*, 2006). The Y181C mutation that results in high-level resistance to NVP and increased susceptibility to AZT was present in 2 of 13 patients. In both patients the mutation occurred with the K103N and A98G mutations. The K101E mutation, which is associated with low-level resistance and the V108I mutation, which on its own is associated with potential low-level resistance, was found in two patients. The clinical impact of this potential low-level resistance is unknown but will probably decrease the threshold for further resistance-associated mutations in the presence of NVP treatment. One of each of the A98G mutation giving low-level resistance to NVP, the V106A mutation giving high-level resistance against NVP, and the G190E mutation also giving high-level resistance to NVP, were found. One patient had the K70R mutation, which results in low-level resistance to AZT. The K70R mutation a thymidine analogue mutations (TAMs) which is the most common mutation occurring in patients receiving AZT for long periods (Chaix *et al*, 2005). These mutations accumulate over time and 4 or more cause high-level resistance to AZT (Ross *et al*, 2001). The patient acquiring this mutation also had a K101E mutation that results in low-level resistance to NVP. The risk of AZT resistance appears to be low since only one patient had a low-level resistance mutation.

The use of a short course of AZT and sd-NVP is effective in lowering the MTCT rate and this study shows that it decreases mutations in woman and increases the

likelihood of successful response to NNRTI containing ART. The Western Cape Province programme has shown that it is feasible to maintain the short course AZT and sd-NVP with 81% of woman being enrolled in the program and a transmission rate of lower than 10% (Coetzee *et al*, 2005).

Like in most studies of sd-NVP, sequencing was used to detect resistance-associated mutations. Since commercial genotypic testing is expensive, the TFIH assay was used. This assay gave a high yield of readable sequences and could be used to look for RT resistance in 97 of 107 (90.7%) patients.

5.1.4 The further optimisation, validation and quality assurance of the in-house HIV-1 antiretroviral resistance tests to be more applicable and practical for routine resistance testing in a diagnostic setting.

Since 2004, South African patients in the public sector have had access to antiretroviral therapy and CD4 and viral load monitoring to detect therapy failure. However as the cost is prohibitive, antiretroviral resistance testing is not part of the HIV management program. Resistance testing is valuable for management of individual patients failing antiretroviral therapy through guiding selection of optimal second line or salvage therapy. It is also necessary for resistance surveillance and as an outcome measure of program success in the South African antiretroviral roll-out. Apart from the high cost, commercial resistance assays are usually designed and validated for HIV-1 subtype B viruses. The sequencing primers do not always perform as well when HIV-1 subtype non-B virus is prevalent (Beddows *et al*, 2003, Fontaine *et al*, 2001).

The manual QIAamp® UltraSens virus kit performed the best of all the extraction kits with a final elution of 60µl. Although the most effective, it is a long and labour intensive method. Therefore, we recommended that for a diagnostic setting the NucliSense EasyMag automated extraction systems with a 25µl elute should be used as this system performed as well as the QIAamp® UltraSens virus kit. Previous studies documented that the NucliSense EasyMag automated extraction systems is as sensitive as manual methods (Perandin *et al*, 2009, Pillet *et al*, 2009, Burgener *et al*, 2003) with a inhibition range at the same level as the QIAamp® UltraSens virus kit (Burgener *et al*, 2003). The efficiency of the NucliSense EasyMag automated

extraction systems were 4 to 6 times higher than the QIAamp® UltraSens virus kit (Burgener *et al,* 2003) but at a lower elution volume.

It was possible to optimise and validate a nested PCR assay, IIH assay, which uses 8 primers for sequencing. A 1200 bp sequence that containing the protease and the 5' of the reverse transcriptase genes where antiretroviral resistance associated mutations are found. The sequence obtained does not cover the *integrase* gene, but integrase inhibitors are not currently used in the South African public sector.

Additives in the PCR reaction are used to enhance the yield and specificity of the reaction. DTT reduces disulfide bonds of proteins (Nagai *et al*, 1998, Ralser *et al*, 2006). In this study the addition of DTT increased the yield cDNA ten-fold in the first round PCR.

The primer annealing temperature is often within 5°C of the melting temperature of the primers. The increase of the annealing temperature closer to or higher than the annealing temperature will lead to increased annealing stringency and minimizes non-specific primer annealing (Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010). In this study, the rise of the annealing temperature closer to the melting temperature in the second round PCR increase the yield of PCR product tenfold.

Too low a specimen input in a PCR reaction can lead to low yields of PCR product and a too high an input can lead to inhibition of the reaction or non specific amplification (Promega, Protocol and Applications guide http://www.promega.com/paguide/chap1.htm, November 2010). The increase of the PCR product input in the second round PCR increase the yield of cDNA product for sequencing.

In this study, a partial and cross-validation of the IIH assay was done as quality control samples and an original validated assay, the ViroSeq™ HIV-1 genotyping assay, serves as a reference.

The analytical sensitivity of the IIH assay was determined to be 137 (87-794; 95%CI) copies/ml. DNA blast of sequences showed that PCR products were specific. A drawback of the study is that we did not evaluate the assay with a full panel of different HIV-1 subtypes and recombinants to test its ability to detect these viruses, although a large number of different subtypes were detected with the assay during its evaluation. However, that should not detract from using the assay.

A second round (nested) PCR reaction allowed us to achieve high analytic sensitivity. As using two rounds of PCR could be more prone to contamination, phylogenetic trees are drawn for each specimen batch, using neighbour-joining phylogenetic trees to identify similar sequences. The *pol* sequences generated were aligned with Clustal X (Thompson *et al*, 1997) and compared with the Kimura two-parameter distance neighbour-joining method to estimate genetic distance (Saitou and Nei, 1987) with 1000 bootstrap replicates in Mega 4.0 software (Kumar *et al*, 2004).The IIH assay also showed good performance on international assessment panels. The performance in the Round Robin HIV-1 genotyping assessment system was better than the ViroSeq™ HIV-1 genotyping assay and TFIH assay, with detection of all but one mutation. Satisfactory results were also obtained with the QCMD evaluations as all mutation present could be detected.

We found the IIH assay more sensitive than a commercial reference assay for detectioning HIV-1 subtype C, producing complete sequencing results on most samples. All 29 samples could be amplified with the IIH assay and good results were obtained from sequencing of the samples with only one incomplete sequence was obtained. The ViroSeq™ HIV-1 genotyping assay could amplify 25 of 29 samples. Incomplete sequences were only obtained in 3 samples; despite the high primer failure rate in more than 80% of the samples. As in the other cases, a complete sequence could be assembled from the remaining sequenced fragments, due to sufficient read-length. This is due to Primer A and D producing a similar sequence. When both failed ould one cnot obtain a complete sequence (Fontaine *et al*, 2001). However the integrity of these results obtained from the assay is compromised as (48%) consensus sequences requiered assembly from single stranded sequencing data. Primer D has previously been shown to perform poorly in non-subtype B samples and other studies previously described a high failure rate of primers A, D and H (Beddows *et al*, 2003, Mracna *et al*, 2001 and Fontaine *et al*, 2001).

The TFIH and IIH assays could amplify 94% and 85% of samples respectively. Although the TFIH assays had a higher amplification rate, it also produced a high number of incomplete sequences (24), which could not be analysed properly for resistance mutations. This could be primer related. New primers were selected for the IIH assay with better results. The IIH assay only produced 4 incomplete sequences. Primer failures occurred in 40% and 32% of samples respectively. The integrity of the

sequences was compromised in only 6% of the samples from the IIH assay. In 79% of samples complete sequence could be detected with the IIH assay. Of the 10 samples failing to amplify with the IIH assay, 3 also did not amplify with the TFIH assay. In 3 samples, only the protease fragment could be amplified. This could possibily be due to the decreased efficiency of the amplification reaction as a result of the larger product size in the IIH assay, which amplifies a 1200 bp target while the two TFIH assay fragments had sizes of 487 and 804 base pairs respectively.

The cost of the IIH assay was R1518.11. There was a decrease in the price of the Ih-house assay of R118.77. The assay could be performed over a 3 day period from sample extraction to the release of the final result.final result.

5.2 Conclusion

We have shown in Chapter 2 that an in-house assay was valuable in monitoring the effect of therapy options on resistance in patients failing therapy and in the monitoring of resistance in therapy naive patients. The extensive optimisation and validation of this assay allowed us to use it with confidence in either a diagnostic or research setting. We obtained SANAS accreditation for its diagnostic use. As the antiretroviral roll out matures, and more patients will have failed both the first and second line regimens there will be an increased need to have affordable resistance tests to select appropriate third-line or salvage regimens.

Chapter 6

6. References

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Appendix A: Protocol from Jacobs *et al* (2008)

Extraction of viral RNA for genotyping

The method used the QIAamp UltraSens Kit (QIAGEN GmbH, Germany) for RNA extraction from 1ml plasma. The manufactures instructions was followed and the RNA was eluted in two elution steps with 30 µl of AVE buffer added to each to obtain a total elution volume of 60 µl.

First round RT-PCR reactions

The Access RT-PCR System (Promega, Madison, USA) was used to do the first round RT-PCR reactions in a one step protocol. The PCR primers are listed in Table A.1 (Plantier *et al*, 2005). Five µl of RNA was added to the first round reaction mix that consisted of AMV/T*fl* 5X reaction buffer at a 1X concentration, a dNTP-mix at 0.2mM each, primers at 40pmol, 2mM $MgSO_2$, AMV RT 0.1U/µl, T*fl* DNA Polymerase 0.1U/µl and nuclease-free water up to a final volume of 50µl. PCR cycling was done using 9700 PCR system thermocyclers (Applied Biosystems, Foster City, USA) at 9600 ramp speed. The reaction mixtures were prepared in 0.2ml PCR tubes (QSP, Porex BioProducts Inc, California, USA).

The following cycling conditions applied: First round PCR: For complementary DNA (cDNA) synthesis reaction tubes were incubated at 48°C for 45 minutes to allow for reverse transcription (yielding double stranded-DNA (ds-DNA)) and then at 94°C for 2 minutes to inactivate AMV Reverse transcriptase and to denature ds-DNA. For exponential amplification of DNA the cycling conditions were: 40 cycles of 94°C for 20 seconds to denature DNA, 55°C for 30 seconds for primer annealing and 68°C for 90 seconds for primer extension. This is followed by a final 5 minutes at 68°C to complete primer extension. The product was then stored at 4°C until the second round reactions could be done.

Table A.1: Primers for genotyping PCR

Protease Primer	**HBX2 number**	**Primer Tm**	**Primer direction**	**Primer Sequence**
outer 30prot1	2703-2734	57.6°C	*Reverse*	5'- GCA AAT ACT GGA GTA TTG TAT GGA TTT TCA GG –3'
outer 50prot1	2082-2109	57.0°C	*Forward*	5'- TAA TTT TTT AGG GAA GAT CTG GCC TTC C -3'
inner 30prot2	2621-2650	55.8°C	*Reverse*	5' AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC –3'
inner 50prot2	2136-2163	68.2°C	*Forward*	5'- TCA GAG CAG ACC AGA GCC AAC AGC CCC A –3'
Reverse transcriptase Primer				
outer Mj4	3399-3420	54.8°C	*Reverse*	5'- CTG TTA GTG CTT TGG TTC CTC T –3'
outer Mj3	2480-2499	54.3°C	*Forward*	5- AGT AGG ACC TAC ACC TGT CA –3'
inner NE13	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3
inner A35	2530-2564	58.4°C	*Forward*	5'- TTG GTT GCA CTT TAA TTC CCA TTA GTC CTA TT –3'

Second round PCR reactions

The *Taq* DNA Polymerase in storage buffer B system (Promega, Madison, USA) was used for the second round PCR reaction mixture using the first round RT-PCR product as template input. The PCR primers used are listed in Table A.1 (Plantier *et al*, 2005). Five µl of the first round RT-PCR reaction product was added to the second round reaction mix which consisted of 5X storage buffer B at a 1X concentration, PCR Nucleotide mix (Promega, Madison, USA) at 0.2mM each, primers at 40pmol each, 1.5mM $MgCl_2$, *Taq* DNA Polymerase at 2.5U/µl, and nuclease-free water up to a final volume of 50µl.

The cycling conditions that applied to the second round PCR were: One cycle at 94°C for 2 minutes for DNA denaturing followed by 40 cycles of 94°C for 20 seconds for DNA denaturing, 55°C for 30 seconds for primers annealing and 68°C for 90 seconds for primer extension to extend. This was followed by a final extension for 5 minutes at 68°C. The product was then stored at 4°C until it could be visualized.

Visualization of the PCR product with agerose gel electrophoresis

The PCR product was identified according to product size (514bp for the protease gene and 804bp for the reverse transcriptase gene) with gel electrophoresis on a 0.8%

agarose gel prepared with LE analytical grade agarose (Whitehead Scientific, Cape Town, RSA) in 1 x TAE Buffer (0.04M Tris acetate, 0.001M EDTA). Five µl of ethydium bromide (Promega, Madison, USA) was added to the gel to intercalate ds-DNA.

Two µl loading buffer (Promega, Madison, USA) was mixed with 5µl PCR product. Each specimen was sequentially loaded in a slot on the gel, including the blanks, positive controls and 1 kilobasepair marker (Promega, Madison, USA). The electrical current was set at 50 mAmperes for a 50ml gel apparatus.

When the products have migrated from the negative to the positive anode sufficiently, as indicated by the migration of the two coloured dyes in the loading buffer, the current was removed and the gel was visualised under ultra violet light at a wavelength of 302nm, ethydium bromide intercalatede DNA (PCR products) fluoresces brightly. A photograph was taken by using the Syngene™ GeneGenius, version 4.00, program (Synoptics Ldt, Cambridge, United Kingdom).

PCR product purification

The QIAquick Spin Kit (QIAGEN GmbH, Germany) was used to clean the PCR products for use in sequencing reactions according to the manufactures instructions.

Sequencing of PCR product

The ABI Prism® BigDye™ Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Warrington, UK) was used for the sequencing reactions. Cycle sequencing reactions were prepared in a 96 well plate by adding 4µl of terminator ready reaction mix to 1µl of template, 1µl of 5pmol primer and 4µl nuclease free water to obtain a final volume of 10µl. The reaction mix was then exposed to thermocycling conditions repeated 25 times with DNA denaturing at 96°C for 10 seconds and primer annealing for 5 seconds at the sequence primer specific annealing temperature. This was followed by a final product extension at 60°C for 4 minutes. After completion of the cycling the reactions were sent to the DNA sequencing facility of Stellenbosch University for sequencing on the ABI 3100 Sequencer (Applied Biosystems, Foster City, USA). The primers used in the sequencing reactions are listed in Table A.2.

Table A.2: Primers used for sequencing of the protease and reverse transcriptase regions with the TFIH assay

PR Primers	**HBX2 number**	**Primer Tm**	**Primer direction**	**Primer Sequence**	**References**
R2051	2486-2504	49.5°C	*Reverse*	5'- TAT RTT GAC AGG TGT AGG T -3'	Personal Communication, John Hackett
pol1D	2251-2271	56.3°C	*Forward*	5'- TCC CTC AAA TCA CTC TTT GGC -3'	Loxton, 2004
JA217	2622-2646	49.5°C	*Reverse*	5'- CTT TTA TTT TTT CTT CTG TCA ATG G -3'	Lindström and Albert, 2003
RT Primers					
pol3D	2869-2884	48.7°C	*Forward*	5' - CAG TAC TGG ATG TGG G - 3'	Loxton, 2004
pol3rev	2882-2901	51.1°C	*Reverse*	5' - CTG AAA AAT ATG CAT CCC CC - 3'	Personal Communication, Susan Engelbrecht
ABB20-3F	2980-2999	51.6°C	*Forward*	5' - ATC AGT ACA ATG TGC TTC CA - 3'	Personal Communication, John Hackett
AK12	2947-2969	50.6°C	*Reverse*	5' - TGG TGT YTC ATT RTT TRY ACT AG - 3'	Lindström and Albert, 2003
AK11	2571-2592	48.4°C	*Forward*	5' - GTA CCA GTA AAA TTA AAR CCA G - 3'	Lindström and Albert, 2003

Assembly and analysis of sequences

Sequencing alignment was done using the Sequencer software V4.7 (Gene Codes Corporation, Ann Arbor USA). The Stanford HIVdb program for Genotyping resistance interpretation was used to detect drug resistance mutations. http://hivdb.stanford.edu/index.html .

Appendix B: Expected mutations as supplied by Lynn Morris from the NICD AIDS Virus Research Unit

Sample	PI Major Resistance Mutations:	PI Minor Resistance Mutations:	NRTI Resistance Mutations:	NNRTI Resistance Mutations:	Insertions
DR10	None	None	M41L E44D D67N T69D V118I M184I L210W T215Y	A98G Y188L G190A	
DR19	None	None	M41LM D67N L210W T215Y	K101E V106M G190A	RT AA Insertion: codon 69 AA: ES NA: GAGTCT
DR28	None	None	M184V T215Y	A98G K103N M230L	
NVP8b			None	K103N	
NVP64			None	V106A G190A	
NVP186			None	K103N V179I G190A	

Appendix C: Expected mutations as supplied by Jochen Bodem (2007) from the Round Robin HIV-1 genotyping assessment system.

Sample	PI Major Resistance Mutations:	PI Minor Resistance Mutations:	NRTI Resistance Mutations:	NNRTI Resistance Mutations:	*Subtype
1	L76V	L33F	M41L	None	Combined viruses
	I84V	A71V	D67K		
	L90M		T69i		
			K70R		
			M184V		
			T215F		
			K219Q		
2	V46I	None	M41L	K103N	B
	V82F		L74I		
			M184V		
			L210W		
			T215Y		
3	None	None	M41L	None	G
			T215Y		
4	G48GV	L10LI	M41LM	K103KN	B
	I54IT	L33F	E44AE	Y188FY	
	V82VA	A71AV	V118I		
	N88NS		L210LW		
	L90LM		T215NSTY		

Appendix C continued: Expected mutations as supplied by Jochen Bodem (2007) from the Round Robin HIV-1 genotyping assessment system

Sample	PI Major Resistance Mutations:	PI Minor Resistance Mutations:	NRTI Resistance Mutations:	NNRTI Resistance Mutations:	*Subtype
5	I54V	L10IV	A62V	K103KN	B
	V82VF	A71V	K65R	V108IV	
	L90M		D67N		
			T69DN		
			V75I		
			F77L		
			F116Y		
			Q151M		
			M184MV		
			K219E		
6	I54V	L10F	M41L	K101E	B
	V82C	V11I	E44DE	V108I	
	I84V	K43T	D67N	G190A	
	L90M	A71T	T69DN		
		G73S	K70R		
		L89V	V75M		
			M184V		
			L210W		
			T215Y		
			K219E		

Appendix D: Final protocol of optimized and validated TFIH assay

Extraction of viral RNA for genotyping

The method used the QIAamp UltraSens Kit (QIAGEN GmbH, Germany) for RNA extraction from 1ml plasma. The manufacturer's instructions supplied with the kit was followed and the RNA was eluted in two elution steps with 30µl of AVE buffer added in each step to obtain a total elution volume of 60µl.

First round PCR reactions

The Access RT-PCR System (Promega, Madison, USA) was used to do the first round PCR reactions in a one step PCR protocol. The PCR primers are listed in Table D.1. Ten µl of RNA was added to the first round reaction mix that consisted of AMV/T*fl* 5X reaction buffer at a 1X concentration, dNTPs mix at 0.2mM each, primers at 40pmol, 2mM $MgSO_2$, AMV RT 0.1U/µl, T*fl* DNA Polymerase 0.1U/µl and nuclease-free water up to a final volume of 50µl. The reaction was done on a 9700 PCR system (Applied Biosystems, Foster City, USA) using the 9600 ramp speed. The reaction mixtures were prepared in 0.2ml PCR tube (QSP, Porex BioProducts Inc, California, USA).

The following cycling conditions were used for first round PCR: Incubation at 65°C for 30 seconds to straighten the RNA was done. The RNA was then cooled to 48°C. Forty µl of the reaction mix was added and for the first strand cDNA synthesis the reaction were incubate for one cycle at 48°C for 45 minutes so that reverse transcription could take place and then a cycle at 94°C for 2 minutes to inactivate AMV Reverse transcriptase and denaturing of the DNA to occur. For second strand cDNA and amplification the reaction was cycled 40 times at 94°C for 20 seconds to denature, at to 55°C for 30 seconds to anneal the primers and at 68°C for 90 seconds to extend the product. A last extension for at 68°C for 5 minutes was done. The reaction was then stored at 4°C until the second round reactions could be done.

Second round PCR reactions

The *Taq* DNA Polymerase in storage buffer B system (Promega, Madison, USA) was used to do the second round PCR reaction mixture using the first round PCR product. The PCR primers used is listed in Table D.1. Two µl of the first round PCR reaction was added two the second round reaction mix that consisted of 5X storage buffer B at a 1X concentration, PCR Nucleotide mix (Promega, Madison, USA) at 0.2 mM each,

primers at 40 pmol each, 1.5 mM $MgCl_2$, *Taq* DNA Polymerase 2.5 U/µl, and nuclease-free water up to a final volume of 50µl.

The following cycling conditions were used for second round PCR reaction mixtures. One cycle at 94°C for 2 minutes for denaturing of the DNA and then cycled for 40 times at 94°C for 20 seconds to denature, at to 55°C for 30 seconds to anneal the primers and at 68°C for 90 seconds to extend the product. A last extension for at 68°C for 5 minutes was done. The reaction was then stored at 4°C until it could be visualized.

Table D.1: Primers used for first and second round PCR

Protease Primer	**HBX2 number**	**Primer Tm**	**Primer direction**	**Primer Sequence**
outer 3'prot1	2703-2734	57.6°C	*Reverse*	5'- GCA AAT ACT GGA GTA TTG TAT GGA TTT TCA GG –3'
outer 5'prot1	2082-2109	57.0°C	*Forward*	5'- TAA TTT TTT AGG GAA GAT CTG GCC TTC C -3'
inner 3'prot2	2621-2650	55.8°C	*Reverse*	5' AAT GCT TTT ATT TTT TCT TCT GTC AAT GGC –3'
inner 5'prot2	2136-2163	68.2°C	*Forward*	5'- TCA GAG CAG ACC AGA GCC AAC AGC CCC A –3'
Reverse transcriptase Primer				
outer Mj4	3399-3420	54.8°C	*Reverse*	5'- CTG TTA GTG CTT TGG TTC CTC T –3'
outer Mj3	2480-2499	54.3°C	*Forward*	5- AGT AGG ACC TAC ACC TGT CA –3'
inner NE13	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3
inner A35	2530-2564	58.4°C	*Forward*	5'- TTG GTT GCA CTT TAA TTC CCA TTA GTC CTA TT –3'

Visualization of the PCR product with electrophoresis

Gel electrophoresis was done to visualize the PCR product on a 1% agarose gel prepared with LE analytical grade agarose (Whitehead Scientific, Cape Town, RSA) in 1 x TAE Buffer (0.04M Tris acetate, 0.001 M EDTA). 50g of agarose were weight in a glass flask and 50ml of 1 x TAE Buffer was added. It was then heated in a microwave oven to boiling point and mixed by stirring on magnetic stirrer. Five µl of ethydium bromide (Promega, Madison, USA) was added to the gel to stain the DNA.

Two µl loading buffer (Promega, Madison, USA) was mixed with 5µl PCR product. Each specimen was sequentially loaded in a slot on the gel, including the blanks,

positive controls and 1 kilobasepair marker (Promega, Madison, USA). A current of 50 mAmperes per 50ml gel was then passed through the gel.

When the products migrated from the negative to the positive anode sufficiently, as indicated by the migration of the two coloured dyes in the loading buffer, the current was removed and the gel was visualised under ultra violet light at a wavelength of 302nm, ethydium bromide stained DNA (PCR products) appeared as bright band. A photograph was taken by using the Syngene™ GeneGenius, version 4.00, program (Synoptics Ldt, Cambridge, United Kingdom).

PCR product purification

The QIAquick Spin Kit (QIAGEN GmbH, Germany) was used to clean the PCR products for use in sequencing reactions according to the manufactures instructions.

Quantification of DNA with the NanoDrop

The DNA product was quantified with the NanoDrop ND-1000. The concentration of DNA obtained was used to work out a dilution factor to obtain 10ng-40ng of DNA to use in the sequencing reactions.

Sequencing of PCR product

The ABI Prism® BigDye™ Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Warrington, UK) was used for the sequencing reactions. Cycle sequencing reactions were prepared in a 96 well plate by adding 4µl of terminator ready reaction mix to 1µl of template, 1µl of 5pmol primer and 4µl nuclease free water to obtain a final volume of 10µl. The reaction mix was then exposed to thermocycling conditions repeated 25 times with DNA denaturing at 96°C for 10 seconds and primer annealing for 5 seconds at the sequence primer specific annealing temperature. This was followed by a final product extension at 60°C for 4 minutes. After completion of the cycling the reactions were sent to the DNA sequencing facility of Stellenbosch University for sequencing on the ABI 3100 Sequencer (Applied Biosystems, Foster City, USA). The primers used in the sequencing reactions are listed in Table D.2.

Table D.2: Primers used for sequencing of the PR and RT region

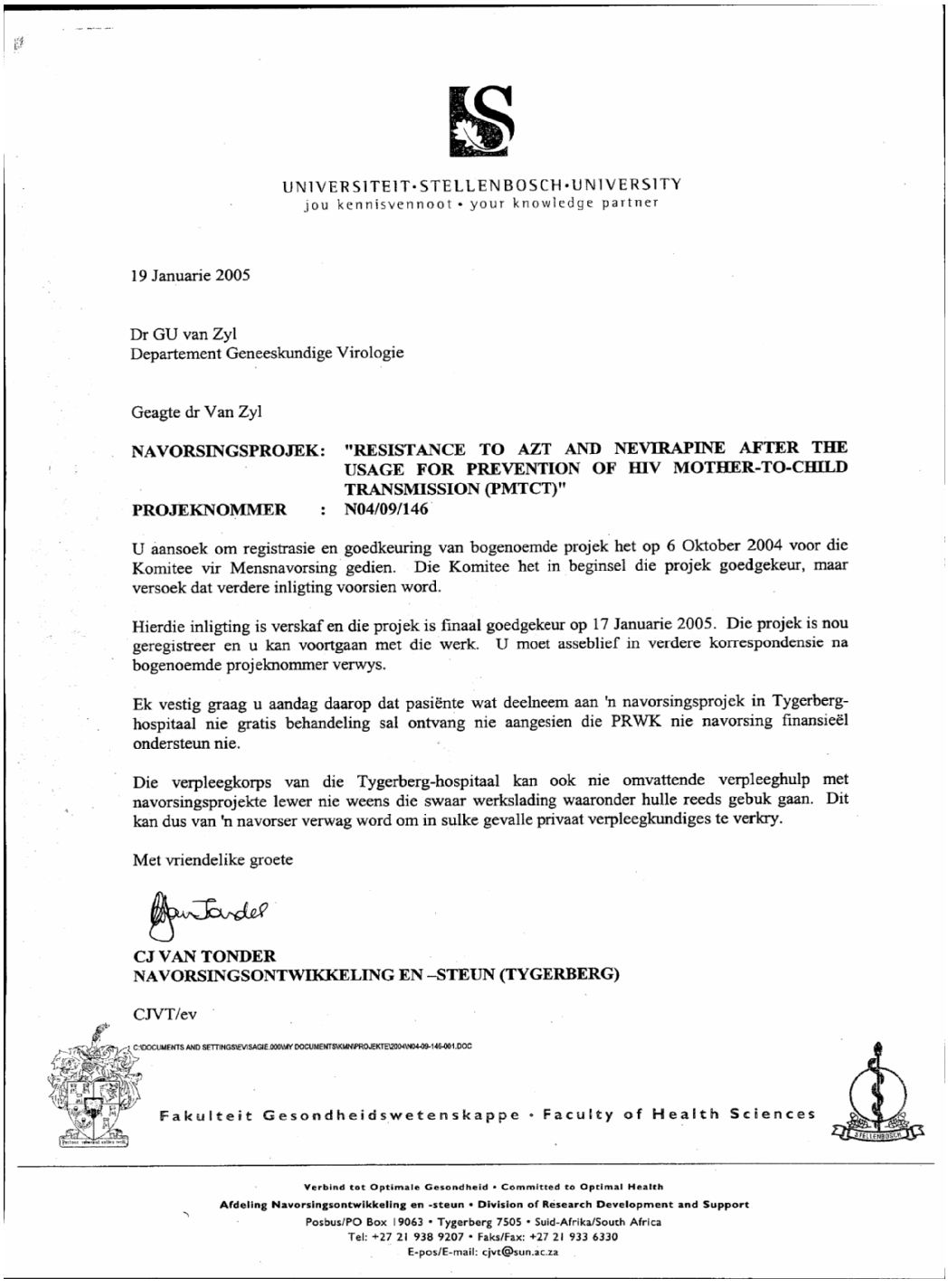
PR Primers	**HBX2 number**	**Primer Tm**	**Primer direction**	**Primer Sequence**	**References**
R2051	2486-2504	49.5°C	*Reverse*	5'- TAT RTT GAC AGG TGT AGG T -3'	Personal Communication, John Hackett
pol1D	2251-2271	56.3°C	*Forward*	5'- TCC CTC AAA TCA CTC TTT GGC -3'	Loxton, 2004
JA217	2622-2646	49.5°C	*Reverse*	5'- CTT TTA TTT TTT CTT CTG TCA ATG G -3'	Lindström and Albert, 2003
RT Primers					
pol3D	2869-2884	48.7°C	*Forward*	5' - CAG TAC TGG ATG TGG G - 3'	Loxton, 2004
pol3rev	2882-2901	51.1°C	*Reverse*	5' - CTG AAA AAT ATG CAT CCC CC - 3'	Personal Communication, Susan Engelbrecht
ABB20-3F	2980-2999	51.6°C	*Forward*	5' - ATC AGT ACA ATG TGC TTC CA - 3'	Personal Communication, John Hackett
AK12	2947-2969	50.6°C	*Reverse*	5' - TGG TGT YTC ATT RTT TRY ACT AG - 3'	Lindström and Albert, 2003
AK11	2571-2592	48.4°C	*Forward*	5' - GTA CCA GTA AAA TTA AAR CCA G - 3'	Lindström and Albert, 2003

Analysis of sequencing results

Sequencing alignment was done using the Sequencer software V4.7 (Gene Codes Corporation, Ann Arbor USA). The Stanford HIVdb program for Genotyping resistance interpretation was used to detect drug resistance mutations. http://hivdb.stanford.edu/index.html,

Appendix E: Letter of approval

Approval for the project was obtained from the Ethics Committee of the University of Stellenbosch (Committee of Human Research) on 17 January 2005. The project number is N04/09/146



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19 Januarie 2005

Dr GU van Zyl
Departement Geneeskundige Virologie

Geagte dr Van Zyl

NAVORSINGSPROJEK: "RESISTANCE TO AZT AND NEVIRAPINE AFTER THE USAGE FOR PREVENTION OF HIV MOTHER-TO-CHILD TRANSMISSION (PMTCT)"
PROJEKNOMMER : N04/09/146

U aansoek om registrasie en goedkeuring van bogenoemde projek het op 6 Oktober 2004 voor die Komitee vir Mensnavorsing gedien. Die Komitee het in beginsel die projek goedgekeur, maar versoek dat verdere inligting voorsien word.

Hierdie inligting is verskaf en die projek is finaal goedgekeur op 17 Januarie 2005. Die projek is nou geregistreer en u kan voortgaan met die werk. U moet asseblief in verdere korrespondensie na bogenoemde projeknommer verwys.

Ek vestig graag u aandag daarop dat pasiënte wat deelneem aan 'n navorsingsprojek in Tygerberg-hospitaal nie gratis behandeling sal ontvang nie aangesien die PRWK nie navorsing finansieël ondersteun nie.

Die verpleegkorps van die Tygerberg-hospitaal kan ook nie omvattende verpleeghulp met navorsingsprojekte lewer nie weens die swaar werkslading waaronder hulle reeds gebuk gaan. Dit kan dus van 'n navorser verwag word om in sulke gevalle privaat verpleegkundiges te verkry.

Met vriendelike groete

CJ VAN TONDER
NAVORSINGSONTWIKKELING EN –STEUN (TYGERBERG)

CJVT/ev

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Appendex F: Publication

Journal of Medical Virology 80:942–946 (2008)

Zidovudine With Nevirapine for the Prevention of HIV Mother-to-Child Transmission Reduces Nevirapine Resistance in Mothers From the Western Cape, South Africa

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In the Western Cape province of South Africa, an intensified regimen for the prevention-of-mother-to-child-transmission-of-HIV consisting of zidovudine (AZT) from 34 weeks of pregnancy plus single dose (sd) nevirapine (NVP) during labor was instituted in 2004. The newborn baby receives a single dose of NVP and AZT for 7 days. Similar strategies in Thailand and Africa have been shown to be more effective in reducing transmission than NVP alone. The use of sd NVP only for the prevention-of-mother-to-child-transmission-of-HIV has a high risk of inducing resistance (25–69%) with an average of 35.7% by a recent meta-analysis and has been shown to adversely affect non-nucleoside reverse transcriptase inhibitor (NNRTI)-based antiretroviral therapy when initiated within 6 months. In this study the prevalence of resistance to NVP and AZT in mothers who had received the intensified regimen was measured. Specimens collected from mothers were genotyped by in-house PCR and sequencing. In specimens obtained within 60 days of delivery, acquired NVP resistance mutations were detected in 13 of 76 patients (17.1%, 95% confidence interval: 8.7–25.6%), which appears to be lower than in studies with sd NVP alone (37.5%, 95% confidence interval: 23.0–50.6%). ***J. Med. Virol. 80:942–946, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HIV; prevention of mother-to-child transmission; nevirapine (NVP); zidovudine (AZT); antiretroviral drug resistance

INTRODUCTION

Antiretroviral drugs are an important component of programs for the prevention-of-mother-to-child-transmission-of-HIV. Single dose (sd) nevirapine (NVP) given to mother and child is the simplest and most affordable prevention-of-mother-to-child-transmission-of-HIV regimen although it is less effective than longer courses of zidovudine (AZT) or combination regimens [Volmink et al., 2007]. Strategies such as the one used in the ACTG076 study, which included AZT orally from a median of 26 weeks of gestation and intravenously intra-partum for the mother and orally for 6 weeks for the neonate, showed a marked reduction in transmission risk [CDC, 1994] but were too complicated and expensive for routine use in most African settings. In Thailand when AZT was started at 28 weeks of gestation combined with sd NVP given in labor, the transmission rate was only 1.9% [Lallemant et al., 2004]. In the DITRAME PLUS [Dabis et al., 2005] and the MASHI [Thior et al., 2006] studies, conducted in Africa, where sd NVP to mother and baby was combined with AZT, given from 36 and 34 weeks gestation,

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respectively, and to the baby for 1 week or 1 month, transmission risks were 6.5% and 5.3%. In comparison, sd NVP alone was associated with a transmission risk of 11.9% in HIVNET 012 [Guay et al., 1999; Jackson et al., 2003].

In 2001, based on evidence from the HIVNET012 study, the Western Cape Province in South Africa established a program using sd NVP for mother and baby which was extended to the whole province by 2003. Following on evidence of efficacy of short course of AZT given to mothers in Thailand from 36 weeks of gestation combined with oral AZT during labor [Shaffer et al., 1999] the regimen was changed in 2004 to the current intensified regimen combining sd NVP during labor with AZT from 34 weeks of pregnancy for mothers not yet qualifying for antiretroviral treatment (ART), that is, with CD4 lymphocyte counts of more than 200/μl and no AIDS-defining illness. The newborn baby receives a single dose of NVP and AZT for 7 days [Eley, 2006].

Sd NVP prevention-of-mother-to-child-transmission-of-HIV carries a high risk of inducing resistance to NVP: various studies using genome sequencing found NVP resistance-associated mutations in 25–69% of patients at 6–8 weeks postpartum [Eshleman et al., 2004, 2005a]. A recent meta-analysis by Arrivé et al. [2007] estimates the risk of resistance at 35.7% (95% confidence interval (CI): 23.0–50.6%). This high rate of resistance may result in an increased risk of failure of non-nucleoside reverse transcriptase inhibitor (NNRTI)-based antiretroviral therapy (ART) regimens in mothers commencing HAART within 6 months of sd NVP, compared to mothers starting later, according to data from an observational cohort in Botswana [Lockman et al., 2007; McConnell et al., 2007], although no such association between time of initiation of HAART and therapy success was found in an earlier study from Thailand where AZT was used in combination with NVP [Jourdain et al., 2004].

The aim of this study was to evaluate the prevalence of NVP and AZT resistance-associated mutations around 6 weeks after delivery in mothers that had received the Western Cape prevention-of-mother-to-child-transmission-of-HIV regimen.

METHODS

Samples were collected from HIV-positive mothers attending antenatal clinics at Tygerberg Academic Hospital, a tertiary center, and Delft Community Clinic, a primary healthcare site within greater Cape Town, between February 2005 and March 2007. The study was approved by Stellenbosch University's Institutional Review Board and informed consent was obtained from all participants. HIV-positive mothers who gave informed consent and were not eligible for HAART or late presentation that did not allow for sufficient counseling to receive HAART, and thus qualified for the Western Cape prevention-of-mother-to-child-transmission-of-HIV regimen, were included.

EDTA blood specimens were collected prior to receiving the prevention-of-mother-to-child-transmission-of-HIV regimen (from 34 weeks of gestation until delivery) and at the closest appointment to 6 weeks after delivery. Viral RNA was extracted from EDTA plasma using the QIAamp UltraSensTM virus kit, according to the manufacturer's protocol (Qiagen, Hilden, Germany).

A method described by Plantier et al. [2005] was adapted to amplify the partial reverse transcriptase-encoding (798 bp) region of the *pol* gene by nested PCR, using the Access RT-PCR system for the first and the GoTaq Flexi Kit (both Promega, Madison, WI) for the second round of amplification. PCR products were purified using the QIAquick kit (Qiagen). Sequencing primers that were used allowed overlapping bidirectional sequencing. Sequencing reactions were performed using the ABI Prism Dye Terminator Cycle sequencing kit on the ABI 3130 DNA Sequencer (both Perkin Elmer, Foster City, CA). Resistance-associated mutations were interpreted using the Stanford University HIV Drug Resistance Database algorithm, available at http://hivdb.stanford.edu. The confidence interval was calculated with a web-based calculator (http://www.dimensionresearch.com/resources/calculators/conf_prop.html).

Since normality could not be assumed, the non-parametric Mann–Whitney *U*-Test was used to compare the mean CD4 counts and log viral load values in the resistant and sensitive groups.

RESULTS

Patients were included for analysis on the basis that both a baseline (prior to receiving the prevention-of-mother-to-child-transmission-of-HIV regimen) and a postpartum follow-up specimen were available. Follow-up specimens were collected from 107 mothers. Mothers were scheduled to be followed up 6 weeks postpartum. Logistical difficulties however resulted in considerable variation in the follow-up interval; the median follow-up time was 7 weeks (range 3–19 weeks). Using the in-house test method sequences could be generated for 97 of 107 follow-up specimens that were adequate to adjudge resistance-associated mutations.

None of the baseline specimens, except one specimen with the A98G mutation, which confers low-level NVP resistance, harbored any NNRTI resistance.

As all 13 specimens that had sequences with NVP resistance mutations had been obtained within the first 60 days postpartum, and thus administration of NVP, only the 76 patients with specimens collected within 60 days postpartum were included in the study, in order not to under-estimate the prevalence of resistance. Therefore the remaining 21 patients were excluded from further analysis. Of the 76 patients included, 62 (82%) had specimens collected between 4 and 8 weeks postpartum (when most other studies recorded NVP resistance).

All baseline sequences, including those from patients who later had NVP resistance mutations, were sensitive

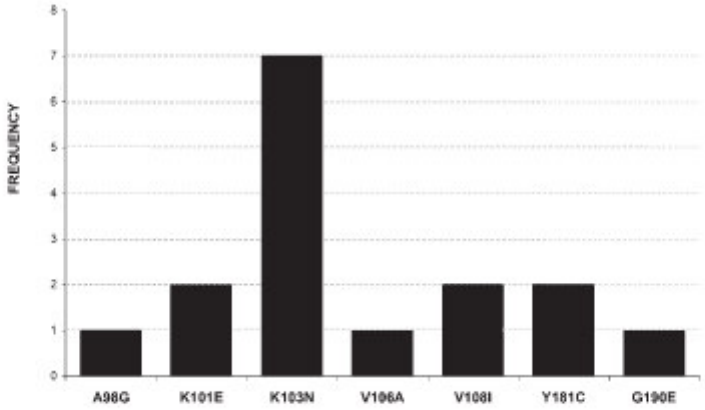

Fig. 1. Frequency of mutations (shown as amino acid positions of the HIV reverse transcriptase) associated with NVP resistance in 76 mothers who had received AZT plus sd NVP for PMTC.

to NVP, except one patient with A98G at baseline, which confers low-level resistance to NVP. In total, 13 out of 76 (17.1%; 95% CI: 8.64–25.5) patients acquired NVP resistance mutations. Of the 13 patients, 9 had high-level NVP resistance (K103N, Y181C, G190E or V106A, alone or in combination with other mutations), 2 had low-level resistance (K101E) and 2 potential low-level resistance (V108I), according to the Stanford HIV Resistance Database Algorithm. The one patient with probable low-level NVP resistance (A98G mutation) in the baseline specimen, in the absence of ever having received NVP, in addition acquired Y181C after NVP exposure.

The mutation most commonly detected was K103N (seven patients). Other NNRTI-associated mutations included K101E, Y181C, V106A, V108I, and G190E. Since 3 of the 13 patients with NVP resistance had 2 mutations, a total of 16 mutations were detected. The frequencies of mutations detected in the 76 patients are shown in Figure 1 and mutations per patient are shown in Table I. Four mothers with no evidence of NVP resistance had previously received NVP for the prevention-of-mother-to-child-transmission-of-HIV, as had one who acquired NVP resistance. In addition, one patient showed the K70R mutation at follow-up, which confers low-level AZT resistance. All sequences were HIV-1 subtype C.

The mean CD4 count (cells per microliter) in the NVP-resistant group was 353 versus 458 in the NVP-sensitive group; a difference which is not statistically significant ($P = 0.07$, Mann–Whitney U-Test). HIV viral load results (VL), performed on the Nuclisens EasyQ assay which has a lower limit of detection of 357 IU/ml for 1 ml input, were available at baseline for 74 of the 76 patients (all 13 patients that had NVP resistance on follow-up and 61 of the 63 patients which remained sensitive). Geometric means of VL for the resistant and sensitive groups respectively were 4,652 (range LDL to 290,000) and 5,250 (range LDL to 330,000) IU/ml. This difference was not statistically significant ($P = 0.94$; Mann–Whitney U-Test for log VL). A comparison of the characteristics of patients that tested sensitive and resistant, respectively, is shown in Table II.

DISCUSSION

The intensified prevention-of-mother-to-child-transmission-of-HIV regimen used in the Western Cape combines AZT with sd NVP. This strategy was selected as it is more effective in lowering the risk of transmission than sd NVP alone. In addition, the addition of AZT is likely to reduce HIV VL prior to administration of sd NVP and could thus reduce the risk of emergence of NVP resistance in mothers receiving the prevention-of-mother-to-child-transmission-of-HIV regimen. To test this hypothesis, we measured the prevalence of NVP resistance in mothers who received the intensified regimen.

TABLE I. Nevirapine (NVP) Resistance-Associated Mutations Detected

Patient	Baseline mutations (n = 1)	Mutations on follow-up (n = 16)	Follow-up interval (days after sd NVP)
1	None	G190E	22
2	None	V108I	55
3	None	K103N	49
4	None	K103N; Y181C	44
5	None	K103N	30
6	None	K103N	20
7	None	K101E	52
8	None	V108I	35
9	None	K103N	34
10	None	K103N; V106A	28
11	None	K103N	44
12	**A98G**	A98G; Y181C	22
13	None	K101E	32

Bold – the only baseline mutation.

TABLE II. Characteristics of Patients That Tested Sensitive and Resistant to NVP on Follow-Up

Parameter	Resistant (n = 13)	Sensitive (n = 63)	*P*-value
Mean CD4 count (range)	353 (168–651) cells per microliter	458 (181–1,018) cells per microliter	0.07
Geometric mean of viral load (range)	4,652 (LDL –290,000)	5,250 (LDL –330,000)	0.94

Like in most studies of sd NVP, sequencing was used to detect resistance-associated mutations. Since commercial genotypic testing is expensive, an in-house method adapted from Plantier et al. [2005] was used. The latter gave a high yield of readable sequences and could be used to adjudge RT resistance in 97 of 107 (90.7%) samples. Although probably less sensitive than allele-specific PCR, this method allowed the detection of less common resistance-associated mutations such as G190E and V106A.

Since all of the patients with NVP resistance mutations, except one patient with A98G at baseline, which confers low-level resistance to NVP, had baseline sequences sensitive to NVP, the resistance mutations detected were enriched by the prevention-of-mother-to-child-transmission-of-HIV intervention. Resistance interpretation was according to the Stanford HIV Resistance Database: In the postpartum follow-up specimens K103N, associated with a high level resistance (48-fold resistance), was the most common resistance mutation (detected in 7 of 13 patients). K101E, which was detected in 2 patients, is associated with a low-level resistance (8.9-fold resistance). Lastly, the V108I mutation, which on its own is associated with potential resistance, a 2.9-fold resistance, was found in two patients. The clinical impact of this "potential resistance" is unknown but will probably decrease the threshold for further resistance-associated mutations in the presence of NVP treatment.

The combined use of AZT and sd NVP for the prevention-of-mother-to-child-transmission-of-HIV probably reduces the risk of NVP resistance since in this study only 13 out of 76 mothers (17.1%; 95% CI: 8.64–25.5) had NVP resistance as detected by PCR and sequencing, compared to settings using sd NVP-only regimens where the average expected resistance prevalence is 37.5% (95% CI: 23.0–50.6%). A potential bias towards under-estimation of resistance in this study is the confounding effect of relatively lower viral load values and higher CD4 counts on resistance prevalence; the reason being that some patients with CD4 counts below 200 cells per microliter were excluded since they qualified for HAART. On the other hand a possible counteracting bias (towards an over-estimation of resistance) could be due to a higher reported risk of NVP resistance in subtype C virus compared to subtypes A and D [Eshleman et al., 2005b]. Comparing patients that tested NVP resistant and sensitive, the CD4 count in the NVP resistant group was lower than in the sensitive group but this difference was not statistically significant ($P = 0.07$). There also was no significant difference in the viral load values between the groups ($P = 0.94$).

Only one specimen had low-level AZT resistance (K70R mutation). Therefore the risk of AZT resistance after this regimen appears to be low.

In summary we detected a prevalence of NVP resistance of 17.1% in patients receiving the Western Cape prevention-of-mother-to-child-transmission-of-HIV intervention. Although this is lower than in cohorts that received sd NVP only, it is still a substantial proportion of those exposed to the intervention.

ACKNOWLEDGMENTS

Sr Lehana Breytenbach collected the clinic information and Dr. Rob Martell of the Provincial Government Western Cape, Department of Health gave permission for the research. Sr Sikiti of the Delft Community Clinic and Mrs Gordon, Facility Manager of the same facility, provided logistic support. Dr. Chris Muller of the Department of Statistics and Actuarial Science, Stellenbosch University, gave advice on statistical analysis.

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Appendix G: Patient characteristics of samples included in the MTCT study

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding [α]Y/[$]N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 1.1	1980/10/15	2005/02/28	LDL	353	22/52	28/06/2005	N	C	[f]S		N
RP 1.2	1980/10/15	2005/08/04						C	S		
RP 7.1	1964/01/09	2005/03/02	14000	286	21/52	26/05/2005	N	C	S		N
RP 7.2	1964/01/09	2005/06/30						C	S		
RP 8.1	1970/09/10	2005/03/04	7900	365	20.4/52	23/07/2006	N	C	S		N
RP 8.2	1970/09/10	2005/08/31						C	S		
RP 9.1	1978/11/07	2005/03/07	1200	624	30/52	31/05/2005	Y	C	S		N
RP 9.2	1978/11/07	2005/06/30						C	S		
RP 10.2	1978/01/04	2005/03/10	5700	717	34/52	19/04/2005	N	C	S		N
RP 10.2	1978/01/04	2005/06/08						C	S		
RP 12.1	1968/02/22	2005/03/14	3200	625	10/52	29/10/2005	N	C	S		N
RP 12.2	1968/02/22	2005/11/04						C	Resist	G190E	
RP 13.1	1980/07/07	2005/03/14	5800	329	31/52	12/05/2005	N	C	S		N
RP 13.2	1980/07/07	2006/07/08						C	S		
RP 17.1	1978/04/12	2005/03/22	5200	344	29/52	03/06/2005	N	C	S		N
RP 17.2	1978/04/12	2005/07/28						C	Resist	V108N	
RP 19.1	1975/01/25	2005/03/24	12000	404	24.5/52	20/07/2005	N	C	S		N
RP 19.2	1975/01/25	2005/08/18						C	S		
RP 25.1	1975/12/08	2005/04/04	6800	357	24/52	17/07/2005	N	C	S		N
RP 25.2	1975/12/08	2005/08/31						C	S		
RP 28.1	1980/11/29	2005/04/04	9300	405	34/52	24/04/2005	N	C	S		N
RP 28.2	1980/11/29	2005/06/08						C	S		
RP 29.1	1976/06/01	2005/04/06	17000	201	34/52	19/05/2005	N	C	S		N
RP 29.2	1976/06/01	2005/07/08						C	Resist	K103KN	

Appendix G continued: Patient characteristics of samples included in the MTCT study

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding Y/N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 35.1	1984/10/10	2005/04/23	20000	733	34/52	06/06/2005	N	C	S		N
RP 35.2	1984/10/10	2005/08/01						C	S		
RP 37.1	1969/08/06	2005/05/10	2000	417	34/52	29/08/2005	N	C	S		N
RP 37.2	1969/08/06	2005/10/05						C	S		
RP 40.1	1973/09/26	2005/06/08	59000	317	29.5/52	12/08/2005	N	C	S		N
RP 40.2	1973/09/26	2005/10/05						C	S		
RP 41.1	1981/12/01	2005/06/28		365	21/52	18/11/2005	N	C	S		N
RP 41.2	1981/12/01	2005/12/05						C	S		
RP 46.1	1975/10/26	2005/09/07	210000	255	36/52	21/10/2006	N	C	S		Yes - 2002
RP 46.2	1975/10/26	2005/12/05						C	Resist	K103KN Y181CY	
RP 47.1	1975/12/06	2005/09/08	29000	297	34/52	23/10/2005	N	C	S		N
RP 47.2	1975/12/06	2005/12/05						C	S		
RP 48.1	1968/09/13	2005/09/08	3200	651	20/52	20/01/2006	N	C	S		N
RP 48.2	1968/09/13	2006/02/20						C	Resist	K103KN T215AT	
RP 49.1	1978/01/19	2005/09/08	8600	737	32/52	02/11/2005	N	C	S		N
RP 49.2	1978/01/19	2005/12/05						C	S		
RP 70.1	1984/12/29	2006/03/08	290000	168	38/52	30/03/2006	N	C	S		N
RP 70.2	1984/12/29	2006/04/20						C	Resist	K103KN	
RP 71.1	1980/11/23	2006/03/13	LDL	502	34/52	14/04/2006	Y	C	S		N
RP 71.2	1980/11/23	2006/06/06						C	Resist	K101EK	
RP 72.1	1985/08/07	2006/03/13	630	595	?28/52	06/06/2006	N	C	S		N
RP 72.2	1985/08/07	2006/07/06						C	S		

Appendix G continued: Patient characteristics of samples included in the MTCT study

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding Y/N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 73.1	1971/02/27	2006/03/13	330000		34/52	17/04/2006	Both	C	S		N
RP 73.2	1971/02/27	2006/05/30						C	S		
RP 74.1	1973/10/28	2006/03/15	LDL	239	?39/52	07/04/2006	N	C	S		N
RP 74.2	1973/10/28	2006/05/12						C	Resist	V108IV	
RP 75.1	1986/02/14	2006/03/22	9000	341	35/52	06/04/2006	N	C	S		N
RP 75.2	1986/02/14	2006/06/01						C	S		
RP 76.1	1980/02/20	2006/03/22	LDL	789	35/52	28/04/2006	N	C	S		N
RP 76.2	1980/02/20	2006/05/30						C	S		
RP 77.1	1978/09/09	2006/03/22	740	233	34/52	11/04/2006	N	C	S		N
RP 77.2	1978/09/09	2006/06/09						C	S		
RP 79.1	1980/11/30	2006/03/22	940	654	34/52	09/04/2006	N	C	S		N
RP 79.2	1980/11/30	2006/06/08						C	S		
RP 83.1	1985/09/21	2006/03/29	1700	443	34/52	23/05/2006	N	C	S		N
RP 83.2	1985/09/21	2006/07/04						C	S		
RP 86.1	1972/05/08	2006/04/03	8600	345	34/52	28/04/2006	N	C	S		N
RP 86.2	1972/05/08	2006/06/15						C	S		
RP 87.1	1982/09/09	2006/04/03	59000	587	37/52	09/04/2006	N	C	S		N
RP 87.2	1982/09/09	2006/06/07						C	S		
RP 88.1	1981/01/21	2006/04/03	1100	390	34/52	02/06/2006	N	C	S		N
RP 88.2	1981/01/21	2006/07/06						C	Resist	K103KN	
RP 91.1	1972/06/16	2006/04/05	54000	510	35/52	28/04/2006	N	C	S		N
RP 91.2	1972/06/16	2006/06/15						C	S		
RP 96.1	1975/10/26	2006/04/19	710	1018	34/52	11/06/2006	N	C	S		N
RP 96.2	1975/10/26	2006/07/11						C	S		

Appendix G continued: Patient characteristics of samples included in the MTCT study

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding Y/N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 99.1	1971/04/21	2006/04/19	29000	243	34/52	06/06/2006	N	C	S		N
RP 99.2	1971/04/21	2006/07/04						C	Resist	K103KN V1O6AV	
RP 100.1	1981/02/06	2006/04/19	36000	553	34/52	09/06/2006	N	C	S		N
RP 100.2	1981/02/06	2006/07/06						C	S		
RP 101.1	1989/02/06	2006/04/24	57000		34/52	03/06/2006		C	S		N
RP 101.2	1989/02/06	2006/07/04						C	S		
RP 105.1	1975/09/28	2006/05/03	5600	365	34/52	21/05/2006	N	C	S		N
RP 105.2	1975/09/28	2006/07/18						C	S		
RP 106.1	1979/01/01	2006/05/03	1500	252	34/52	31/05/2006	N	C	S		N
RP 106.2	1979/01/01	2006/07/18						C	S		
RP 107.1	1977/06/03	2006/05/03	11000	289	34/52	25/05/2006	N	C	S		N
RP 107.2	1977/06/03	2006/07/20						C	S		
RP 111.1	1981/10/06	2006/05/15	34000	181	35/52	05/06/2006	N	C	S		N
RP 111.2	1981/10/06	2006/07/18						C	S		
RP 113.1	1981/09/21	2006/05/15	510	697	34/52	07/07/2006	N	C	S		N
RP 113.2	1981/09/21	2006/08/25						C	S		
RP 116.1	1984/10/22	2006/05/22	5100	603	34/52	30/06/2006	N	C	S		N
RP 116.2	1984/10/22	2006/08/15						C	S		
RP 121.1	1984/07/30	2006/05/29	17000	226	34/52	07/06/2006	N	C	S		N
RP 121.2	1984/07/30	2006/08/01						C	S		
RP 122.1	1974/05/01	2006/06/05	14000	261	34/52	08/07/2006	N	C	S		N
RP 122.2	1974/05/01	2006/08/22						C	Resist	K103KN	
RP 124.1	1971/06/24	2006/06/05	46000	655	34/52	13/07/2006	N	C	S		N
RP 124.2	1971/06/24	2006/08/22						C	S		

Appendix G continued: Patient characteristics of samples included in the MTCT study

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding Y/N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 126.1	1978/05/16	2006/06/07	5400	518	34/52	18/07/2006	N	C	S		N
RP 126.2	1978/05/16	2006/08/29						C	S		
RP 128.1	1984/06/28	2006/06/12	29000	316	34/52	25/07/2006	N	C	S		N
RP 128.2	1984/06/28	2006/09/12						C	S		
RP 129.1	1979/06/15	2006/06/12	25000	300	34/52	17/07/2006	N	C	S		N
RP 129.2	1979/06/15	2006/08/29						C	S		
RP 131.1	1976/02/15	2006/06/19	510	658	34/52	21/07/2006	N	C	S		Yes - 2004
RP 131.2	1976/02/15	2006/08/22						C	S		
RP 133.1	1979/11/24	2006/06/14	1400	738	34/52	01/08/2006	N	C	S		Yes - 2004
RP 133.2	1979/11/24	2006/09/14						C	S		
RP 134.1	1978/02/21	2006/06/19	14000	188	34/52	13/07/2006	N	C	S		N
RP 134.2	1978/02/21	2006/08/25						C	S		
RP 135.1	1986/11/26	2006/06/19	6300	209	34/52	11/07/2006	N	C	S		N
RP 135.2	1986/11/26	2006/08/29						C	S		
RP 138.1	1980/02/24	2006/06/26	13000	384	34/52	27/09/2006	N	C	S		N
RP 138.2A	1980/02/24	2007/06/26			34/53	27/09/2007	N	C	S		N
RP 138.2B	1980/02/24	2006/10/31						C	S IC		
RP 139.1	1988/02/14	2006/06/26	4000	335	34/52	07/08/2006	N	C	P R	A98G	N
RP 139.2	1988/02/14	2006/08/29						C	Resist	A98G Y181CY	
RP 140.1	1973/05/15	2006/06/28	1400	625	34/52	28/06/2006	N	C	S		N
RP 140.2	1973/05/15	2006/08/24						C	S		
RP 143.1	1985/12/20	2006/06/28	29000	248	34/52	31/08/2006	N	C	S		N
RP 143.2	1985/12/20	2006/09/28						C	S		

Appendix G continued: Patient characteristics of samples included in the MTCT study

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding Y/N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 144.1	1982/09/16	2006/07/10	2600	597	34/52	05/08/2006	N	C	S		N
RP 144.2	1982/09/16	2006/09/14						C	S IC		
RP 145.1	1971/01/15	2006/07/10	63000	342	34/52	16/08/2006	N	C	S		N
RP 145.2	1971/01/15	2006/09/14						C	S		
RP 149.1	1984/01/25	2006/07/19	12000	613	34/52	30/08/2006		C	S		N
RP 149.2	1984/01/25	2006/09/28						C	S		
RP 150.1	1983/10/29	2006/07/09	1400	564	34/52	22/09/2006	N	C	S		N
RP 150.2	1983/10/29	2006/10/26						C	S		
RP 151.1	1979/05/13	2006/07/19	1700	428	34/52	28/08/2006		C	S		N
RP 151.2	1979/05/13	2006/09/28						C	S		
RP 152.1	1987/05/06	2006/07/19	14000	206	34/52	30/08/2006		C	S		N
RP 152.2	1987/05/06	2006/09/28						C	S		
RP 156.1	1983/07/04	2006/07/26	1000	587	34/52	30/08/2006		C	S		N
RP 156.2	1983/07/04	2006/09/27						C	S		
RP 157.1	1985/11/10	2006/07/26	2600	498	34/52	06/09/2006		C	S		N
RP 157.2	1985/11/10	2006/10/05						C	S		
RP 159.1	1983/05/30	2006/08/16	9700	380	35/52	24/09/2006	N	C	S		N
RP 159.2	1983/05/30	2006/10/26						C	Resist	K70KR K101EK	
RP 162.1	1991/08/14	2006/08/30	2000	204	34/52	04/10/2006	N	C	S IC		N
RP 162.2	1991/08/14	2006/11/07						C	S IC		
RP 165.1	1975/01/01	2006/09/11	65000	878	34/52	13/10/2006	N	C	S		N
RP 165.2	1975/01/01	2006/11/21						C	S		
RP 166.1	1982/05/27	2006/09/13	3600	261	34/52	19/10/2006	N	C	S		N
RP 166.2	1982/05/27	2006/11/23						C	S		

Appendix G continued: Patient characteristics of samples included in the MTCT study.

Sample Code	Date of Birth	Date of Sampling	Viral load (RNA Copies/ml)	Baseline CD4 (Cells/µl)	Week of pregnancy	Date of Delivery	Breast feeding [α]Y/[$]N	HIV-1 Subtype	*Resist	Mutations	Previous MTCT? Y/N
RP 167.1	1984/03/09	2006/09/13	3300	334	34/52	08/10/2006	N	C	S IC		N
RP 167.2	1984/03/09	2006/11/09						C	S IC		
RP 169.1	1985/10/22	2006/09/13	6900	526	34/52	27/11/2006	N	C	S		N
RP 169.2	1985/10/22	2007/01/19						C	S		
RP 172.1	1975/10/11	2006/09/18		295	34/52	25/09/2006	N	C	S		Yes - 2005
RP 172.2	1975/10/12	2006/11/17						C	S		
RP 173.1	1969/08/11	2006/06/20	12000	372	34/52	14/10/2006	N	C	S		Yes - 2004
RP 173.2	1969/08/11	2006/11/09						C	S		
RP 178.1	1981/09/05	2006/10/18	3400	364	34/52	29/11/2006	N	B	S		No
RP 178.2	1981/09/05	2007/01/10						B	S		

[α]Y = Yes

[$]N = No

[*]Resist = Resistant

[f]S = Sensitivity

Appendix H: Final protocol for Improved In-house assay

Extraction of viral RNA for genotyping

The method used the QIAamp UltraSens Kit (QIAGEN GmbH, Germany) for RNA extraction from 1ml plasma. The manufactures instructions supplied was followed and the RNA was eluted in two elution steps with 30µl of AVE buffer added to each step to obtain a total elution volume of 60µl.

First round PCR reactions

The Access RT-PCR System (Promega, Madison, USA) was used to do the first round PCR reactions in a one step PCR protocol. The PCR primers used is listed in Table H.1. Ten microliters of RNA was added to the first round reaction mix that consisted of AMV/T*fl* 5X reaction buffer at a 1X concentration, dNTPs mix at 0.2mM each, primers at 40pmol, 2mM $MgSO_2$, AMV RT 0.1U/µl, T*fl* DNA Polymerase 0.1U/µl, DTT at 0.8mM and nuclease-free water up to a final volume of 50µl. The reaction was done on a 9700 PCR system ((Applied Biosystems, Foster City, USA) using the 9600 ramp speed. The reaction mixtures were prepared in 0.2ml PCR tube (QSP, Porex BioProducts Inc, California, USA).

The following cycles was used for first round PCRs. Incubation at 65°C for 30 seconds to denature the RNA was done. The RNA was then cooled to 48°C. Forty microliter of the reaction mix was added and for the first strand cDNA synthesis the reaction were incubate for one cycle at 48°C for 45 minutes so that reverse transcription could take place and then a cycle at 94°C for 2 minutes to inactivate AMV Reverse transcriptase and denaturation of the DNA to occur. For second strand cDNA and amplification the reaction was cycled 40 times at 94°C for 20 seconds to denature, at to 55°C for 30 seconds to anneal the primers and at 68°C for 90 seconds to extend the product. A last extension for at 68°C for 5 minutes was done. The reaction was then stored at 4°C until the second round reactions could be done.

Table H.1: Primers used for IIH assays genotyping PCR

First round Primers	HBX2 number	Primer Tm	Primer direction	Primer Sequence
outer 5'prot1	2082-2109	57.0°C	*Forward*	5'- TAA TTT TTT AGG GAA GAT CTG GCC TTC C -3'
outer Mj4	3399-3420	54.8°C	*Reverse*	5'- CTG TTA GTG CTT TGG TTC CTC T –3'
Second Round Primers				
inner 5'prot2	2136-2163	68.2°C	*Forward*	5'- TCA GAG CAG ACC AGA GCC AAC AGC CCC A –3'
inner NE135	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3

Second round PCR reactions

The *Taq* Go-Taq® Flexi DNA polymerase (Promega, Madison, USA) was used to do the second round PCR reaction mixture using the first round PCR product. The PCR primers used is listed in Table H.1. Two µl of the first round PCR reaction was added two the second round reaction mix that consisted of 5X buffer at a 1X concentration, PCR Nucleotide mix (Promega, Madison, USA) at 0.2mM each, primers at 40pmol each, 2mM $MgCl_2$, *Taq* DNA Polymerase 2.5U/µl, and nuclease-free water up to a final volume of 25µl.

The following cycles was used for second round PCR reaction mixtures. One cycle at 94°C for 2 minutes for denaturing of the DNA and then cycled for 40 times at 94°C for 20 seconds to denature, at to 60°C for 30 seconds to anneal the primers and at 68°C for 90 seconds to extend the product. A last extension for at 68°C for 5 minutes was done. The reaction was then stored at 4°C until it could be visualized.

Visualization of the PCR product with electrophoresis

Gel electrophoresis was done to visualize the PCR product on a 1% agarose gel prepared with LE analytical grade agarose (Whitehead Scientific, Cape Town, RSA) in 1 x TAE Buffer (0.04M Tris acetate, 0.001M EDTA). 500mg of agarose were weight in a glass flask and 50ml of 1 x TAE Buffer was added. It was then microwave to boiling point and mixed by stirring on magnetic stirrer. Five µl of ethydium bromide (Promega, Madison, USA) was added to the gel to stain the DNA.

Two µl loading buffer (Promega, Madison, USA) was mixed with 5µl PCR product. Each specimen was sequentially loaded in a slot on the gel, including the blanks,

positive controls and 1 kilobasepare marker (Promega, Madison, USA). A current of 50 mAmperes per 50ml gel was then passed through the gel.

When the products migrated from the negative to the positive anode sufficiently, as indicated by the migration of the two coloured dyes in the loading buffer, the current was removed and the gel was visualised under ultra violet light at a wavelength of 302nm, ethydium bromide stained DNA/PCR products appeared as bright band. A photograph was taken by using the Syngene™ GeneGenius, version 4.00, program (Synoptics Ldt, Cambridge, United Kingdom).

PCR product purification

The QIAquick Spin Kit (QIAGEN GmbH, Germany) was used to clean the PCR products for use in sequencing reactions according to the manufactures instructions.

Quantification of DNA with the NanoDrop

The DNA product was quantified with the NanoDrop ND-1000. The concentration of DNA obtained was used to work out a dilution factor to obtain 10ng-40ng of DNA to use in the sequencing reactions.

Sequencing of PCR product

The ABI Prism® BigDye™ Terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Warrington, UK) was used for the sequencing reactions. Cycle sequencing reactions were prepared in a 96 well plate by adding 1µl of terminator ready reaction mix to 3µl of sequencing buffer, 1µl of template, 1µl of 5pmol primer and 4µl nuclease free water to obtain a final volume of 10µl. The reaction mix was then exposed to thermocycling conditions repeated 25 times with DNA denaturing at 96°C for 10 seconds and primer annealing for 5 seconds at the sequence primer specific annealing temperature. This was followed by a final product extension at 60°C for 4 minutes. After completion of the cycling the reactions were sent to the DNA sequencing facility of Stellenbosch University for sequencing on the ABI 3100 Sequencer (Applied Biosystems, Foster City, USA). The primers used in the sequencing reactions are listed in Table H.2.

Table H.2: Primers used for IIH assays sequencing

Primer	HBX2 number	Primer Tm	Primer Direction	Primer Sequence	References
pol1D	2254-2271	56.3°C	*Forward*	5'- TCC CTC AAA TCA CTC TTT GGC -3'	Loxton, 2004
F2054	2511-2528	53.4°C	*Forward*	5' – GGR CGA AAT ATG TTG ACT CAG – 3'	Personal Communication, John Hackett
AK11	2574-2592	48.4°C	*Forward*	5' - GTA CCA GTA AAA TTA AAR CCA G - 3'	Lindström and Albert, 2003
JA217	2622-2643	49.5°C	*Reverse*	5'- CTT TTA TTT TTT CTT CTG TCA ATG G -3'	Lindström and Albert, 2003
pol 3	2885-2901	63.4°C	*Forward*	5' - GGG GGA TGC ATA TTT TTC AG –3'	Personal Communication, Susan Engelbrecht
inner NE135	3300-3334	61.4°C	*Reverse*	5'- CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT –'3	Plantier *et al*, 2005
ABB20-3F	2983-2999	51.6°C	*Forward*	5' – ATC AGT ACA ATG TGC TTC CA - 3'	Personal Communication, John Hackett
AK10	3316-3333	50.5°C	*Reverse*	5' - TYC CCA CTA AYT TCT GTA TRT C –3'	Lindström and Albert, 2003

Alignment and analysis of sequencing results

Sequencing alignment was done using the Sequencer software V4.7 (Gene Codes Corporation, Ann Arbor USA). The Stanford HIVdb program for Genotyping resistance interpretation was used to detect drug resistance mutations. http://hivdb.stanford.edu/index.html, Updated January 2007. The *pol* sequences generated were aligned with Clustal X (Thompson et al., 1997) and compared with the Kimura two-parameter distance neighbour-joining method (Saitou and Nei, 1987) with 1000 bootstrap replicates in Mega 4.0 software (Kumar 2004). The subtype *pol* reference sequences provided by Los Alamos National Laboratory (http://www.hiv.lanl.gov/content/index) were included in the phylogenetic tree.

Appendix J: Patient characteristics of samples used for comparison of the IIH assay with the Viroseq™ HIV-1 genotyping system

Sample	Age (years)	Gender	Specimen Date	Treatment	*Subtype
330314	9	[$]M	14/12/09	Unknown	C
330315	1	M	14/12/09	Unknown	C
330316	12	M	11/12/09	3TC,D4T,EFV	C
330318	42	[α]F	11/12/09	3TC,TDF,EFV	C
330818	33	F	17/12/09	Unknown	C
330819	15	F	17/12/09	Unknown	C
330820	15	F	17/12/09	3TC,D4T,KLT,ABC	C
330822	6	F	15/12/09	Unknown	C
330823	7	M	15/12/09	3TC,D4T,EFV	C
331101	9	F	18/12/09	Unknown	C
331502	42	F	18/12/09	3TC,AZT,EFV	C
332071	7	M	22/12/09	3TC,D4T,EFV	C
332885	62	F	31/12/09	3TC,D4T,EFV	C
333599	15	M	07/01/10	3TC,D4T,EFV	C
333601	4	F	05/01/10	3TC,D4T,EFV	C
333676	36	F	07/01/10	EFV,COMBIVIR	C
333679	46	F	07/01/10	3TC,EFV,TDF	C
333983	11	M	08/01/10	3TC,D4T,EFV	C
333985	4	M	08/01/10	3TC,AZT,EFV,DDI	C
334791	6	F	11/01/10	Unknown	C
335286	14	F	14/01/10	3TC,D4T,EFV	C
335287	36	F	14/01/10	Unknown	C
335291	15	F	14/01/10	3TC,ABC,EFV	C
335294	47	M	13/01/10	Unknown	C
335299	34	M	13/01/10	3TC,AZT,EFV	C
335301	14	F	14/01/10	3TC,D4T,EFV	C
336514	7	M	15/01/10	3TC,D4T,EFV	D
336517	33	F	13/01/10	3TC,D4T,NVP	C
336844	5	F	20/01/10	AZT,ABC,RTV,	C

*The Rega HIV-1 subtyping tool Version 2.0 (http://dbpartners.stanford.edu/Rega_Subtyping/) was used for determining the HIV-1 subtype

[$]M = Male

[α]F = Female

Appendix I: Resistance mutations detected with Viroseq™ HIV-1 genotyping system and IIH assay

Viroseq™ HIV-1 genotyping system						**IIH assay**					
Sample	**Mutations**					**Sample**	**Mutations**				
	PR Major	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**		**PR Major**	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**
330314	None	None	M184V	K103N H221HY M230L	C	330314	None	None	M184V	K103N H221HY M230L	C
330315						330315	L24I L33F M46I I54V V82A	L10F 71V	L74V Y115F M184V	None	C
330316	None	L10LV	M184V	K103R V106M V179D	C	330316	None	L10LV	M184V	K103R V106M V179D	C
330318			K70EK M184V	A98G K103N P225H	C	330318	None	T74S	K70EK M184V	A98G K103N P225H	C
330818	None	A71T	A62AV D67DN M184V T215Y	V106M E138EK F227L	C	330818	None	A71T	A62AV D67DN V75IV M184V T215STY	V106M E138EK F227L	C
330819	None	None	None	None	C	330819	None	None	None	None	C
330820						330820	None	None	M184V	None	C
330822	None	None	M184V	K101E E138S G190A	C	330822	None	None	M184V	K101E E138S G190AG	C

Appendix I continued: Resistance mutations detected with Viroseq™ HIV-1 genotyping system and IIH assay

Viroseq™ HIV-1 genotyping system						**IIH assay**					
Sample	**Mutations**					**Sample**	**Mutations**				
	PR Major	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**		**PR Major**	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**
330823						330823	None	T74S	A62AV M184V	V106M E138A V179D F227FL	C
331101	None	T74S	M184V	V106M V179DV F227L	C	331101	None	T74S	M184V	V106M F227L	C
331502	None	T74S	M184V	K103N E138A P225H	C	331502	None	T74S	M184V	K103N E138A P225H	C
332071	None	None	T69ST M184V	K101E V106M G190A	C	332071	None	None	M184V L210IL	K101E V106M G190A	C
332885	None	None	M184V	K103N	C	332885	None	None	M184V	K103N	C
333599	None	T74S	D67N M184V	K103N	C	333599	None	T74S	D67N M184V	K103N	C
333601	None	None	M184V	V106M F227L	C	333601	None	None	M184V	V106M F227L	C
333676	None	None	M184V L210W T215Y	A98G K103N P225H	C	333676	None	None	M184V L210W T215Y	A98G K103N P225H	C
333679	None	None	None	K103KN V179S	C	333679	None	None	None	K103KN	C

Appendix I continued: Resistance mutations detected with Viroseq™ HIV-1 genotyping system and IIH assay

Viroseq™ HIV-1 genotyping system						**IIH assay**					
Sample	**Mutations**					**Sample**	**Mutations**				
	PR Major	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**		**PR Major**	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**
333983	None	T74S	M184V	K101Q V106M G190AG F227L	C	333983	None	T74S	M184V	K101Q V106M G190A F227L	C
333985	None	T74S	K70R M184 K219E	K101N V106M Y181C	C	333985	None	T74S	D67DN K70R M184V K219E	K101N V106M Y181C	C
334791	None	None	M184V	K101P K103N	C	334791	None	None	M184V	K101P K103N E138EK	C
335286	None	None	M184V	K101E V106M G190A	C	335286	None	None	M184V	K101E V106M G190A	C
335287	None	T74S	T69N M184V	K103N	C	335287	None	T74V	T69N K70R M184V K219KQ	K101KPQT K103N	C
335291	None	Q38E	L41IL M184V	V106M V179D	C	335291	None	Q38E	L41IL M184V	V106M V179DV G190AG	C
335294	None	T74S	None	E138A	C	335294	None	T74V	None	E138A	C

Appendix I continued: Resistance mutations detected with Viroseq™ HIV-1 genotyping system and IIH assay

Viroseq™ HIV-1 genotyping system						**IIH assay**					
Sample	**Mutations**					**Sample**	**Mutations**				
	PR Major	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**		**PR Major**	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**
335299	None	None	M184V	K103N P225H	C	335299	None	None	M184V	K103N P225H	C
335301						335301	None	None	M184MV	K101E V106M V108I G190A	C
336514	None	None	M184V	K101Q K103N P225H	D	336514	None	A71AT	M184V	K101Q K103N P225H	D
336517						336517	None	None	M184V	K103N	C
336844	None	L10V	None	K101E Y181C G190A	C	336844	None	L10LV	None	K101E Y181C G190A	C

Appendix K: Discrepant mutations between Viroseq™ HIV-1 genotyping system and IIH assay

Viroseq™ HIV-1 genotyping system						**IIH assay**				
Sample	**Mutations**					**Mutations**				
	PR Major	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**	**PR Major**	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**
330315						L24I L33F M46I I54V V82A	L10F A71V	L74V Y115F M184V	None	C
330818	None	A71T	A62AV D67DN M184V T215Y	V106M E138EK F227L	C	None	A71T	A62AV D67DN V75IV M184V T215STY	V106M E138EK F227L	C
330820						None	None	M184V	None	C
330823						None	T74S	A62AV M184V	V106M E138A V179D F227FL	C
331101	None	T74S	M184V	V106M V179DV F227L	C	None	T74S	M184V	V106M F227L	C
332071	None	None	T69ST M184V	K101E V106M G190A	C	None	None	M184V L210IL	K101E V106M G190A	C
333679	None	None	None	K103KN V179S	C	None	None	None	K103KN	C

Appendix K continued: Discrepant mutations between Viroseq™ HIV-1 genotyping system and IIH assay

Viroseq						**IIH assay**				
Sample	**Mutations**					**Mutations**				
	PR Major	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**	**PR Major**	**PR Minor**	**NRTI**	**NNRTI**	**Subtype**
333985	None	T74S	K70R M184 K219E	K101N V106M Y181C	C	None	T74S	D67DN K70R M184V K219E	K101N V106M Y181C	C
334791	None	None	M184V	K101P K103N	C	None	None	M184V	K101P K103N E138EK	C
335287	None	T74S	T69N M184V	K103N	C	None	T74V	T69N K70R M184V K219KQ	K101KPQT K103N	C
335291	None	Q38E	L41IL M184V	V106M V179D	C	None	Q38E	L41IL M184V	V106M V179DV G190AG	C
335301						None	None	M184MV	K101E V106M V108I G190A	C
336514	None	None	M184V	K101Q K103N P225H	D	None	A71AT	M184V	K101Q K103N P225H	D
336517						None	None	M184V	K103N	C

Appendix L: Patient characteristics of samples used for TFIH assay and IIH assay comparison

Sample	Age in years	Gender	Specimen Date	Treatment
144745	6	[α]F	2008/01/14	Unknown
144771	38	F	2008/01/15	3TC,AZT,EFV
145426	7	[$]M	2008/01/21	3TC, LPV/r,ABC
145427	9	M	2008/01/21	3TC,d4T,EFV
145831	41	M	2008/01/22	LPV/r,AZT,ddi
145881	55	F	2008/01/17	LPV/r,AZT,ddi
145882	34	F	2008/01/17	3TC,d4T,EFV
146142	15	M	2008/01/24	3TC, LPV/r,ABC,AZT
146143	54	F	2008/01/24	3TC,d4T,EFV
146593	5	F	2008/01/28	3TC,d4T,KLT
147028	27	F	2008/01/30	LPV/r,AZT,ddi
147642	36	F	2008/01/31	3TC,d4T,NVP
147643	41	F	2008/01/31	LPV/r,AZT,ddi
148090	6	M	2008/02/05	3TC,d4T,EFV
148091	7	M	2008/02/05	3TC,d4T,EFV
148092	3	Unknown	2008/02/05	3TC,d4T,LPV/r
148093	3	F	2008/02/05	3TC,d4T,LPV/r
148221	8	Unknown	2008/02/06	3TC,d4T,LPV/r
148222	33	F	2008/02/06	3TC,AZT,EFV
148824	41	M	2008/02/08	3TC,AZT,EFV
148825	12	M	2008/02/08	Unknown
149004	12	M	2008/02/11	3TC,KLT,ABC
149517	42	M	2008/02/13	LPV/r,AZT,ddi
149518	18	F	2008/02/12	Unknown
150142	34	F	2008/02/14	Unknown

Appendix L continued: Patient characteristics of samples used for TFIH assay and IIH assay comparison

Sample	Age in years	Gender	Specimen Date	Treatment
150143	41	M	2008/02/15	3TC,AZT,EFV
151267	9	F	2008/02/20	3TC,AZT,ddi
151450	37	F	2008/02/21	3TC,d4T,EFV
151679	52	F	2008/02/19	Unknown
151680	7	F	2008/02/19	Unknown
151924	12	F	2008/02/22	3TC,d4T, LPV/r,ABC
151925	8	F	2008/02/26	3TC,d4T,EFV
152122	43	M	2008/02/27	3TC,d4T,EFV
152529	51	M	2008/02/28	3TC,d4T.ddi
152869	6	F	2008/02/13	3TC,d4T,EFV
153545	45	M	2008/03/06	3TC,d4T,EFV
153547	42	F	2008/03/06	3TC,AZT,EFV
153549	49	M	2008/03/05	Unknown
153550	45	F	2008/03/05	LPV/r,AZT,ddi
153551	40	F	2008/03/05	3TC,AZT,EFV
153792	37	M	2008/03/07	3TC,AZT,EFV
153793	44	F	2008/03/07	3TC,d4T,EFV
Aretas 12	3	M	2007/05/15	AZT,3TC,EFV
Aretas 19	8	M	2007/05/15	ddI,ABC, LPV/r
Aretas 20	6	M	2007/05/08	d4T,3TC,EFV
Aretas 21	57	F	2007/07/18	d4T, 3TC, EFV
Aretas 22	1	F	2007/06/07	d4T, 3TC, RTV
Aretas 23	13	F	2007/06/20	AZT,3TC,NVP
Aretas 24	2	M	2007/06/06	d4T,3TC, NVP
Aretas 25	7	F	2007/07/12	AZT,3TC, NVP

Appendix L continued: Patient characteristics of samples used for TFIH assay and IIH assay comparison

Sample	Age in years	Gender	Specimen Date	Treatment
Aretas 29	2	F	2007/07/26	AZT,3TC, LPV/r
Aretas 30	2	M	2007/08/07	d4T, 3TC, KLT, RTV
Aretas 32	2	F	2007/07/16	d4T,3TC, LPV/r
Aretas 43	13	M	2007/08/20	d4T,3TC, ddi
Aretas 84	5	M	2008/01/21	AZT,3TC,EFV
Aretas 88	6 months	F	2008/01/30	None
Aretas 89	35	F	2008/01/31	d4T, 3TC, NVP
Aretas 90	4 months	M	2008/01/15	None
Aretas 91	1	M	2008/02/01	None
Aretas 92	4 months	M	2008/02/05	None
Aretas 93	11 months	M	2008/01/15	None
Aretas 94	4	M	2008/02/06	d4T, 3TC, LPV/r
Aretas 95	7	F	2007/11/28	d4T, 3TC, EFV
Aretas 100	26	M	2008/01/14	d4T, 3TC, EFV
Aretas101	32	M	2008/02/07	d4T, 3TC, NVP
Aretas 102	1	F	2008/03/06	None

[$]M = Male
[α]F = Female

Appendix M: Comparison of mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
144745	M184V	K103N Y188H	None	None	C	M184V	K103N Y188H	None	None	C
144771	M41L D67N K70N V75T T215Y K219KN	V106M Y188CFL	None	None	C	M41L D67N K70N V75T T215Y K219KN	V106M Y188CFL	None	None	C
144831	None	K103N	None	None	C	None	K103N	None	None	C
145426	No Amp	No Amp	None	None	C	No Amp	No Amp	No Amp	No Amp	
145427	None	None	None	T74S	C	None	None	None	T74S	C
145881	No Amp	No Amp	None	None	C	No Amp	No Amp	No Amp	No Amp	
145882	None	K103N P225HP L234IL	None	None	B	None	K103N P225HP L234IL	None	None	B
146142	No Amp	No Amp	None	L10I	C	No Amp	No Amp	No Amp	No Amp	
146143	No Amp	No Amp	No Amp	No Amp		No Amp	No Amp	No Amp	No Amp	
146593	M184V	None	None	None	C	V75L M184V	None	None	None	C
147028	D67G K70KR M184V K219EK	L100I K103N	L76LIV	None	C	D67G	L100I K103N	L76LIV	None	C
147642	M184V	K103N V179L Y181C	None	None	C	M184V	K101E Y181C G190A	None	None	C

Appendix M continued: Comparison of mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
147643	None	None	None	T74TS	C	None	None	None	None	C
148090	M184V	K103 P225H	None	None	C	M184V	K103 P225H	None	None	C
148091	M184V	K103N V108I	None	None	C	No Amp	No Amp	No Amp	No Amp	
148092	M184V	None	None	L10I	C	M184V	None	None	L10I	C
148093	T69S	Y181C	None	None	C	T69S	Y181C	None	None	C
148221	M184V	None	No Amp	No Amp	C	M184V	None	None	None	C
148222	None	None	None	None	C	No Amp	No Amp	No Amp	No Amp	
148824	K65KR D67N M184IMV	K101E	M46MI V82L L90LM	None	C	K65KR D67N M184IMV	None	M46MI V82L L90LM	None	C
148825	M184V T215Y	K103N	None	None	C	M184V T215Y	K103N	None	None	C
149004	M41L M184V	V106M V179T G190A F227L	None	None	C	M41L M184V	V106M V179T G190A F227L	None	None	C
149517	None	K103KN	None	None	C	None	None	None	None	C
149518	M41L D67N K70N V75T T215Y K219N	V106M Y188*L F227L	None	None	C	M41L E44DE D67N K70N V75T T215Y K219N	V106M Y188L F227L	None	None	C

Appendix M continued: Comparison of mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
150142	M184V	None	None	None	C	M184V	None	None	None	C
150143	No Amp	No Amp	No Amp	No Amp		No Amp	No Amp	No Amp	No Amp	
151267	D67N T215F	K101P K103N	No Amp	No Amp	C	D67N T215F	K101P K103N	I54V V82A	L10LI	C
151450	M184V	Y188L	None	None	C	M184V	A98AG Y188L	None	None	C
151679	M184V	K101E V106M G190AG F227FL	None	None	C					
151680	D67G	Y106M Y188C	None	T74TS	C	D67G	Y106M Y188C	None	None	C
151924	Q151KLMQ M184V	K103N	No Amp	No Amp	C	F116FY Q151KLM Q M184V	K103N	E35G	None	C
151925	A62V M184V	K101H V106M F227FL	None	None	C	M184V	K101H V106M G190A	None	None	C
152122	M184V	V106M G190A	None	None	C	M184V	V106M G190A	None	None	C
152529	None	K103N	No Amp	No Amp	C	None	K103KN	None	None	C
152869	M184V	K101E V106M F227L	No Amp	No Amp	C	M184V	K101E V106M F227L	None	None	C
153545	V118I	None	None	None	C	E44EV V118I	None	None	None	C

Appendix M continued: Comparison of mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
153547	No Amp	No Amp	None	None	C	M184V	K103N V106M	None	None	C
153549	None	None	None	None	B	None	None	None	None	B
153550	None	K103N	None	None	C	None	K103N	None	None	C
153551	None	None	None	None	C					
153792	D67N T69N K70N M184V T215NSTY K219E	K101P K103N	None	None	C	D67N T69N K70N M184V T215Y K219E	K101P K103N	None	None	C
153793	M184V T215IT	K103N F227FL M230L	M46V	None	C	M184V T215IT	K103N M230L	M46V	None	C
Aretas 12	M184V	K103N P225H	None	None	C	M184V	K103N P225H	None	None	C
Aretas 19	M41L	A98AG K101E	No Amp	No Amp	C	M41L M184V T215Y	A28G K101E G190A	None	None	C
Aretas 20	M184V	K103N E138K Y188L	None	None	C	M184V	K103N E138K Y188L	None	None	C
Aretas 21	M184V	K103N V108I P225H	None	None	C	M184V	K101KN K103N V108I P225H	None	None	C
Aretas 22	M184V	None	None	T74S	C	M184V	None	None	T74S	C

Appendix M continued: Comparison of mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
Aretas 23	M41L M184V L210W T215Y	A28G K101E G190A	None	None	C	M41L M184V L210W T215Y	A28G K101E G190A	N88NK	None	C
Aretas 25	T69N M184V	V108IV Y181C	None	None	C	T69N M184V	V108IV Y181C	None	None	C
Aeretas 24	M184V	Y181C	None	T74S	C	T69S M184V	V108IV Y181C	None	T74S	C
Aeretas 29	M184V	None	None	None	C	M184V	None	I54IV	None	C
Aeretas 30	V75L M184V	None	None	None	C	V75L M184V	None	None	None	C
Aretas 32	No Amp	No Amp	No Amp	No Amp		M184V	None	I54V V82A	L10I T74S	C
Aretas 43	D67N M184I T215F	L100I K100E K103N	None	None	C	D67N M184I T215F	L100I K100E K103N	None	None	C
Aretas 84	D67N T69NT K70R M184V K219Q	A98G K101E K103R G190A	None	None	C	D67N T69N K70R M184V T215NSTY K219Q	A98G K101E K103R G190A	None	None	C
Aretas 88	None	None	None	None	C	None	None	None	None	C
Aretas 89	M184V	G190A	None	None	C	V75I M184V	G190A	None	None	C
Aretas 90	T69S	None	None	None	C	T69S	None	None	None	C
Aretas 91	None	None	None	None	C	V75I	None	None	None	C

Appendix M continued: Comparison of mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
Aretas 92	None	None	None	None	C	None	None	None	None	C
Aretas 93	None	None	None	None	C	None	None	None	None	C
Aretas 94	M184V	None	M46I I54V V82A	L10LF L24I	C	M184V	None	M46I I54V V82A	L10F L24I	C
Aretas 95	V118I	None	None	None	C	V118I	None	None	None	C
Aretas 96	No Amp	No Amp	No Amp	No Amp		No Amp	No Amp	No Amp	No Amp	
Aretas 100	None	K103KN	No Amp	No Amp	C	None	K103KN	None	None	C
Aretas 101	V118FV M184V	V108IV Y181C	None	None	C	M184V	V106MV Y181C	None	None	C
Aretas 102	None	None	None	None	C	None	None	None	None	C

Appendix N: Comparison of discrepant mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
146593	M184V	None	None	None	C	V75L M184V	None	None	None	C
147028	D67G K70KR M184V K219EK	L100I K103N	L76LIV	None	C	D67G	L100I K103N	L76LIV	None	C
147643	None	None	None	T74TS	C	None	None	None	None	C
149517	None	K103KN	None	None	C	None	None	None	None	C
149518	M41L D67N K70N V75T T215Y K219N	V106M Y188L F227L	None	None	C	M41L E44DE D67N K70N V75T T215Y K219N	V106M Y188L F227L	None	None	C
151450	M184V	Y188L	None	None	C	M184V	A98AG Y188L	None	None	C
151680	D67G	Y106M Y188C	None	T74TS	C	D67G	Y106M Y188C	None	None	C
151925	A62V M184V	K101H V106M F227FL	None	None	C	M184V	K101H V106M G190A	None	None	C
153545	V118I	None	None	None	C	E44EV V118I	None	None	None	C
153793	M184V T215IT	K103N F227FL M230L	M46V	None	C	M184V T215IT	K103N M230L	M46V	None	C

Appendix N continued: Comparison of discrepant mutations between TFIH assay and the IIH assay

	TFIH assay					IIH assay				
Sample	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**	**NRTI**	**NNRTI**	**Major PI**	**Minor PI**	**Subtype**
Aretas 21	M184V	K103N V108I P225H	None	None	C	M184V	K101KN K103N V108I P225H	None	None	C
Aretas 23	M41L M184V L210W T215Y	A28G K101E G190A	None	None	C	M41L M184V L210W T215Y	A28G K101E G190A	N88NK	None	C
Aeretas 24	M184V	Y181C	None	T74S	C	T69S M184V	V108IV Y181C	None	T74S	C
Aeretas 29	M184V	None	None	None	C	M184V	None	I54IV	None	C
Aretas 84	D67N T69NT K70R M184V K219Q	A98G K101E K103R G190A	None	None	C	D67N T69N K70R M184V T215NST Y K219Q	A98G K101E K103R G190A	None	None	C
Aretas 89	M184V	G190A	None	None	C	V75I M184V	G190A	None	None	C
Aretas 91	None	None	None	None	C	V75I	None	None	None	C
Aretas 101	V118FV M184V	V108IV Y181C	None	None	C	M184V	V106MV Y181C	None	None	C